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Standard Operating Procedures and Field Methods Used for Conducting Ecological Risk Assessment Case Studies

**Naval Construction Battalion
Center Davisville, RI, and Naval
Shipyard Portsmouth, Kittery, ME**

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Standard Operating Procedures and Field Methods Used for Conducting Ecological Risk Assessment Case Studies

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ADMINISTRATIVE INFORMATION

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This document was prepared by the Marine Environmental Support Office (MESO) of NRaD and has been reviewed by NCCOSC NRaD and ERL-N and approved for publication. Approval does not signify that the contents reflect the views and policies of the U. S. Navy or the U. S. Environmental Protection Agency, nor does mention of trade names of commercial products constitute endorsement or recommendation for use.

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We wish to thank Al Haring, Jim Szykman, Linda Dietz, and Kristen Wall of Northern Division of Naval Facilities Engineering Command; Lou Fayan of Naval Construction Battalion Center Davisville; Jim Tayon of Naval Shipyard Portsmouth and the members of the Technical Review Committees for Naval Construction Battalion Davisville and Naval Shipyard Portsmouth for their ongoing support and contributions. Thanks also to Allison Currie of UNH, JEL, and Roy Fransham of MESO for preparation of the final document.

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FOREWORD

The emphasis on determining the ecological impacts of hazardous substances on coastal and estuarine ecosystems requires the use of appropriate methods and procedures to obtain accurate and comparable data. The methods and procedures presented in this document have been field-tested during research and monitoring activities performed to support ecological risk assessment case studies. The case studies were developed as part of an interagency Memorandum of Agreement between the U. S. Navy Naval Command, Control and Ocean Surveillance Center (NCCOSC) Research, Development, Test, and Evaluation Division (NRaD), San Diego, CA, and the U. S. Environmental Protection Agency (EPA) Environmental Research Laboratory Narragansett (ERL-N), Narragansett, RI. The case studies included a marine ecological risk assessment pilot study for Naval Construction Battalion Center, Davisville, RI, and an estuarine ecological risk assessment for Naval Shipyard Portsmouth, Kittery, ME.

The methods and procedures documented in this report were prepared by investigators at ERLN, University of New Hampshire Jackson Estuarine Laboratory (UNH, JEL), and the Marine Environmental Support Office (MESO) of NCCOSC NRaD who were involved in particular aspects of the case studies. The methods were applied within an ecological risk assessment framework to evaluate their applicability to characterize ecological risk. This document has been prepared to fully document the procedures used in the case studies and to assist in the development of suitable techniques capable of achieving the objectives of ecological monitoring and assessment activities. As more information is developed, these procedures will provide a basis for improving and expanding the capabilities needed to accurately assess ecological risk.

We hope this document helps meet the crucial need to establish standard operation procedures (SOPs) for environmental sampling. Anthropogenic effects on our environment are ubiquitous, but are difficult to quantify because there is no clear record of environmental parameters prior to man's influence, and where some data exist, sampling methods are poorly defined. Because of recent losses in natural resources attributable to man, numerous monitoring programs are being established. These programs range in scale from national to local: U. S. EPA's Environmental Monitoring and Assessment Program (national), Gulf of Maine Council on the Marine Environment (regional), Massachusetts Bays Program (state), Gray Bay Watch, NH, and Rhode Island Pond Watchers (local). SOPs will allow data collected by various programs and separated by large scales of space and time to be compared and combined to answer important questions regarding changes in natural resources. The ability to share data will be especially useful in developing relationships between human activities and natural resources that will guide resource management.

Once established, these procedures are not carved in stone, but through the iterative trial and error process that is part of science, should be improved. Continued development of these procedures is anticipated. The value of establishing these 'early' procedures is that they provide a foundation for improvement as well as providing a clear record of

the procedures used in the sampling. Thus, these initial measures will be able to be compared to future measurements using an intercalibration of established and better procedures.

The value of data is not assured unless standard laboratory practices are coupled with a quality assurance and quality control (QA/QC) program suitable for the sample protocol. This is a system of internal checks on all aspects of sample collection and analysis that could compromise results. The resources expended on long-term monitoring are too great to ignore this prerequisite for acceptability of data into large regional databases. The contact person for each of these SOPs was responsible for developing or applying the method to the project, and has developed a QA/QC protocol, available upon request. The contact person is also available to answer specific questions regarding the SOPs.

This document has been organized into three sections. Section 1 was prepared by researchers at EPA-ERL and consists of SOPs for general laboratory and field methods, and chemistry methods. General laboratory and field methods includes procedures for collecting and preparing samples, measuring sediments and water column attributes, culturing test organisms and conducting bioassays. The chemistry methods provides procedures for the analysis of trace levels contaminants (subparts per billion range for some organic compounds and subparts per million range for metals) suitable for a wide range of environmental assessment activities. Specific SOPs are provided for sample collection and storage, preparation for organic and trace metal analysis, analysis of samples by a variety of instrumentation, and instrument maintenance. Procedures for generic activities, such as sampling plan development and data management, currently are not incorporated in this manual.

Section 2 was prepared by researchers at UNH, JEL and provides procedures used for sampling and analysis of estuarine habitats. Section 3, prepared by MESO, documents procedures for sampling and analysis of ultratrace levels of organotin compounds in seawater, sediment, and tissue samples. Appendix A provides results of organotin analysis optimization techniques to obtain subpart per trillion detection levels in seawater and part per billion detection levels in tissues and sediments. Appendix B contains a detailed description of analytical procedures for extracting organotin compounds from soft tissues of marine organisms. A complete guidance document for conducting field and laboratory quality assurance and quality control (QA/QC) protocols, criteria, and corrective action for the Estuarine Ecological Risk Assessment for Naval Shipyard Portsmouth is included in appendix C.

A uniform format, incorporating aspects of SOP layouts used by other organizations, has been adopted throughout this document. This format consists of a descriptive title, the date of SOP creation or revision, and a point of contact (POC) for communication regarding the procedure. The POC is either the original developer of the method, or is the individual currently most familiar with the method. Following this information are six sections that provide (I) an **OBJECTIVE** statement regarding the purpose and use of the method; (II) a list of **MATERIALS AND EQUIPMENT** needed to conduct the activity; (III) the actual **METHODS** used in the procedure; (IV) suggestions and procedures for **TROUBLESHOOTING** the method; (V) information regarding **STATISTICAL**

PROCEDURES AND DATA USAGE; and (VI) a list of bibliographic REFERENCES referring to original published descriptions and supporting documentation. Various sub-sections are included under these heading as appropriate. A primary goal in SOP development was to incorporate sufficient detail within each section such that a reasonably technically oriented user could successfully perform the method with no additional information. Questions which do arise, however, should be addressed to the POC.

Document control data for each SOP are given in four lines located in the upper right-hand corner of each page. The first line contains the SOP number which is reflected in the contents for the manual. The second line provides the Revision Number for the current SOP. The date of this revision is given in the third line. Pagination and the total number of pages in the SOP are provided in the fourth line. Questions regarding individual SOPs can be directed by referencing the relevant document control data.

This SOP manual will be maintained as a "living document." Individual descriptions will be updated in a continuous fashion as advances in scientific understanding of biological, chemical, and physical processes are incorporated into assessment procedures. Major procedural changes that potentially invalidate previous SOP approaches will be noted where appropriate. Additionally, new SOPs will be incorporated into the manual as they are finalized. Updated versions of specific SOPs may be obtained by contacting the Technical Information Manager of the developing laboratory.

CONTENTS

SECTION 1: ERL-N SOPs

ERL-N SOP 1.01.001 Revision 0	
ERL-N Standard Operating Procedure Clean Room Maintenance	1
ERL-N SOP 1.01.002 Revision 0	
ERL-N Standard Operating Procedure Histological Preparation for Shellfish and Fish	7
ERL-N SOP 1.01.003 Revision 0	
ERL-N Standard Operating Procedure Culturing <i>Cyprinodon variegatus</i> , <i>Menidia beryllina</i> , <i>Mysidopsis bahia</i> , and <i>Arbacia punctulata</i>	12
ERL-N SOP 1.01.004 Revision 0	
ERL-N Standard Operating Procedure Preparation of Hypersaline Brine from Natural Seawater	25
ERL-N SOP 1.01.005 Revision 0	
ERL-N Standard Operating Procedure Sediment Grain Size Analysis	28
ERL-N SOP 1.01.006 Revision 0	
ERL-N Standard Operating Procedure General Data Entry	33
ERL-N SOP 1.01.007 Revision 0	
ERL-N Standard Operating Procedure Cell Subculture Methods	35
ERL-N SOP 1.02.001 Revision 0	
ERL-N Standard Operating Procedure Benthic Organism Collection	38
ERL-N SOP 1.02.002 Revision 0	
ERL-N Standard Operating Procedure Caged Bivalve Deployment	42
ERL-N SOP 1.02.003 Revision 0	
ERL-N Standard Operating Procedure Mysid Cage Construction and Field Deployment	46
ERL-N SOP 1.02.004 Revision 0	
ERL-N Standard Operating Procedure Suspended Solids Determination in Water Samples	54
ERL-N SOP 1.02.005 Revision 0	
ERL-N Standard Operating Procedure Water Column Salinity, Conductivity, Temperature, and Dissolved Oxygen Determination	57
ERL-N SOP 1.03.001 Revision 0	
ERL-N Standard Operating Procedure <i>Champia parvula</i> Sexual Reproduction Test	59
ERL-N SOP 1.03.002 Revision 0	
ERL-N Standard Operating Procedure Conducting Acute Toxicity Tests Using <i>Ampelisca abdita</i>	72

ERL-N SOP 1.03.003 Revision 0	
ERL-N Standard Operating Procedure Conducting Acute Toxicity Tests Using <i>Mysidopsis bahia</i> <i>Cyprinodon variegatus</i> , and <i>Menidia beryllina</i>	87
ERL-N SOP 1.03.004 Revision 0	
ERL-N Standard Operating Procedure Conducting the Inland Silverside (<i>Menidia beryllina</i>) and Sheepshead Minnow (<i>Cyprinodon variegatus</i>) Larvae Survival and Growth Tests	94
ERL-N SOP 1.03.005 Revision 0	
ERL-N Standard Operating Procedure Conducting the <i>Mysidopsis bahia</i> Survival, Growth, and Fecundity Test	112
ERL-N SOP 1.03.006 Revision 0	
ERL-N Standard Operating Procedure Conducting the Sea Urchin, <i>Arbacia punctulata</i> , Fertilization Test	125
ERL-N SOP 1.03.007 Revision 0	
ERL-N Standard Operating Procedure Conducting the Sea Urchin Larval Development Test	135
ERL-N SOP 1.03.008 Revision 0	
ERL-N Standard Operating Procedure Coot Clam (<i>Mulinia lateralis</i>) Embryo/Larval Toxicity Test	150
ERL-N SOP 1.03.009 Revision 0	
ERL-N Standard Operating Procedure Microtox	168
ERL-N SOP 1.03.010 Revision 0	
ERL-N Standard Operating Procedure Performing the Phagocytic Index and Killing Ability Assay	169
ERL-N SOP 1.03.011 Revision 0	
ERL-N Standard Operating Procedure Solid Phase Bioaccumulation Testing	173
ERL-N SOP 1.03.012 Revision 0	
ERL-N Standard Operating Procedure V79/Sister Chromatid Exchange Assay	177
ERL-N SOP 1.03.013 Revision 0	
ERL-N Standard Operating Procedure Growth and Scope for Growth Measurements with <i>Mytilus edulis</i>	189
ERL-N SOP 1.03.014 Revision 0	
ERL-N Standard Operating Procedure Membrane Filtration Method for the Enumeration of <i>Clostridium perfringens</i> in Marine Waters	218
ERL-N SOP 1.03.015 Revision 0	
ERL-N Standard Operating Procedure Membrane Filtration Method for the Enumeration of Enterococci in Marine Waters	222
ERL-N SOP 1.03.016 Revision 0	
ERL-N Standard Operating Procedure Membrane Filtration Method for the Enumeration of Fecal Coliforms and <i>Escherichia coli</i> in Marine Waters	226

ERL-N SOP 1.03.017 Revision 0	
ERL-N Standard Operating Procedure Most Probable Number Method for the Enumeration of <i>Clostridium perfringens</i> in Marine Waters	230
ERL-N SOP 1.03.018 Revision 0	
ERL-N Standard Operating Procedure Most Probable Number Method for the Enumeration of Enterococci in Marine Waters	234
ERL-N SOP 1.03.019 Revision 0	
ERL-N Standard Operating Procedure Most Probable Number Method for the Enumeration of Fecal Coliforms and <i>Escherichia Coli</i> in Marine Waters .	239
ERL-N SOP 1.03.020 Revision 0	
ERL-N Standard Operating Procedure Measurement of the Ecological Effects, Fate, and Transport of Chemicals in a Site—Specific Experimental Marine Microcosm	245
ERL-N SOP 1.03.21 Revision 0	
ERL-N Standard Operating Procedure ETC Biological Test Procedures for <i>Ampelisca abdita</i>	266
ERL-N SOP 2.01.001 Revision 0	
ERL-N Standard Operating Procedure Cleaning of Equipment for Trace Metal Analysis	276
ERL-N SOP 2.01.002 Revision 0	
ERL-N Standard Operating Procedure Field Use of the High Volume Seawater Sampling Apparatus for Organics Analysis	278
ERL-N SOP 2.02.002 Revision 0	
ERL-N Standard Operating Procedure Subtidal Sediment Chemistry Sampling	282
ERL-N SOP 2.03.001 Revision 0	
ERL-N Standard Operating Procedure Extraction of Filter Samples for PCBs	285
ERL-N SOP 2.03.002 Revision 0	
ERL-N Standard Operating Procedure Extraction of Sediment Samples for PCBs	288
ERL-N SOP 2.03.003 Revision 0	
ERL-N Standard Operating Procedure Extraction for Tissue Samples	291
ERL-N SOP 2.03.004 Revision 0	
ERL-N Standard Operating Procedure Extraction of Water Samples for PCBs	294
ERL-N SOP 2.03.005 Revision 0	
ERL-N Standard Operating Procedure Column Chromatography of Semivolatile Organic Analytes	297
ERL-N SOP 2.03.006 Revision 0	
ERL-N Standard Operating Procedure Digestion of Organism Samples for Trace Metal Analysis	300

ERL-N SOP 2.03.007 Revision 0	
ERL-N Standard Operating Procedure Microwave Digestion of Organism Samples for Inorganic Analysis	303
ERL-N SOP 2.03.008 Revision 0	
ERL-N Standard Operating Procedure Preparation of Water Samples for Direct Determination of Trace Metals	307
ERL-N SOP 2.03.009 Revision 0	
ERL-N Standard Operating Procedure Extraction of Seawater Samples for Organic Analysis	309
ERL-N SOP 2.03.010 Revision 0	
ERL-N Standard Operating Procedure Sediment Extraction for Semivolatile Organic Analytes	311
ERL-N SOP 2.03.011 Revision 0	
ERL-N Standard Operating Procedure Tissue Extraction for Semivolatile Organic Analytes	314
ERL-N SOP 2.03.012 Revision 0	
ERL-N Standard Operating Procedure Total Microwave Digestion of Sediment Samples for Inorganic Analysis	316
ERL-N SOP 2.03.013 Revision 0	
ERL-N Standard Operating Procedure Ultrasonic Extraction of Sediment Samples for Inorganic Analysis	320
ERL-N SOP 2.04.001 Revision 0	
ERL-N Standard Operating Procedure Analysis of Dissolved PCBs Using Foam Plugs	323
ERL-N SOP 2.04.002 Revision 0	
ERL-N Standard Operating Procedure Gas Chromatography-Mass Spectrometry	326
ERL-N SOP 2.04.003 Revision 0	
ERL-N Standard Operating Procedure Gas Chromatography	331
ERL-N SOP 2.04.004 Revision 0	
ERL-N Standard Operating Procedure Inorganic Analysis by Flame Atomic Absorption Spectrophotometry	335
ERL-N SOP 2.04.005 Revision 0	
ERL-N Standard Operating Procedure Inorganic Analysis by ICP	338
ERL-N SOP 2.04.006 Revision 0	
ERL-N Standard Operating Procedure Instrumental Operating Conditions for Inorganic Analysis	343
ERL-N SOP 2.05.001 Revision 0	
ERL-N Standard Operating Procedure Dichlovos Monitoring	345
ERL-N SOP 2.05.002 Revision 0	
ERL-N Standard Operating Procedure Propoxur Monitoring	347

ERL-N SOP 2.05.003 Revision 0

ERL-N Standard Operating Procedure Carbaryl Monitoring	350
--	-----

SECTION 2: UNH SOPs

JEL SOP 1.01 Revision 0

UNH, JEL Standard Operating Procedure Eelgrass (<i>Zostera marina</i>) Collection and Population Characteristics	353
--	-----

JEL SOP 1.02 Revision 0

UNH, JEL Standard Operating Procedure Eelgrass (<i>Zostera marina</i>) Carbon, Nitrogen, and Phosphorus	360
---	-----

JEL SOP 1.03 Revision 0

UNH, JEL Standard Operating Procedure Seaweed Collection and Population Characteristics	365
---	-----

JEL SOP 1.04 Revision 0

UNH, JEL Standard Operating Procedure Blue Mussel (<i>Mytilus edulis</i>) Collection and Population Characteristics	367
---	-----

JEL SOP 1.05 Revision 0

UNH, JEL Standard Operating Procedure Water Sampling for Suspended Solids Chlorophyll, pH and Nutrients	370
---	-----

JEL SOP 1.06 Revision 0

UNH, JEL Standard Operating Procedure Water Sample Filtration and Analysis of Total Suspended Solids, Chlorophyll and Phaeopigments	372
---	-----

JEL SOP 1.07 Revision 0

UNH, JEL Standard Operating Procedure Analysis of Seawater Samples for Ammonium (NH_4^+) Using Wet Chemistry Procedure	378
---	-----

JEL SOP 1.08 Revision 0

UNH, JEL Standard Operating Procedure Analysis of Seawater Samples for Phosphate (PO_4^{3-}) Using Wet Chemistry Procedure	381
---	-----

JEL SOP 1.09 Revision 0

UNH, JEL Standard Operating Procedure Most Probable Number Method for the Enumeration of <i>Clostridium perfringens</i> in Marine Sediments	384
---	-----

JEL SOP 1.10 Revision 0

UNH, JEL Standard Operating Procedures Sediment Coring, Core Extrusion and Subsampling	388
--	-----

JEL SOP 1.11 Revision 0

UNH, JEL Standard Operating Procedure Sediment Textural Analysis	392
--	-----

JEL SOP 1.12 Revision 0

UNH, JEL Standard Operating Procedure Analysis of Seawater Samples for Nitrate and Nitrite (NO_3^- , NO_2^-) Using an Automated Procedure	396
---	-----

JEL SOP 1.13 Revision 0

NAI Standard Operating Procedure Winter Flounder (<i>Pleuronectes americanus</i>) and Lobster (<i>Homarus americanus</i>) Collection for Chemical Analysis	400
--	-----

JEL SOP 1.14 Revision 0	
NAI Standard Operating Procedure Collection of Sediment Samples for Chemical and Toxicological Analyses, and Characterization of Benthos	402
JEL SOP 1.15 Revision 0	
NAI Standard Operating Procedure Sorting and Identification of Benthic Invertebrates	404

SECTION 3: MESO SOP

MESO SOP February 1992	
MESO Standard Operating Procedure Analysis of Organotin Compounds in Water, Sediment, and Tissue	409

APPENDIX A: Optimizitation of Butyltin Measurements for Seawater, Tissue, and Marine Samples	A-1
---	-----

APPENDIX B: Analytical Procedures for Extractable Organotins in Soft Tissues of Marine Organisms	B-1
---	-----

APPENDIX C: Analytical Chemistry Quality Assurance and Quality Control Protocols, Criteria, and Corrective Action for the Estuarine Ecological Risk Assessment at Naval Shipyard Portsmouth, Kittery, ME	C-1
--	-----

**ERL-N STANDARD OPERATING PROCEDURE
CLEAN ROOM MAINTENANCE**

**ERL-N SOP 1.01.001
REVISION 0
DECEMBER 1990
PAGE 1 OF 6**

POINT OF CONTACT:

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I. OBJECTIVE

Daily and periodical maintenance procedures of the clean room and incubators must be made for maintaining a clean environment for testing and maintenance of tissue cultures.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Clean room environment maintenance.

- Disposable medium lab coats - Arnold David Cohen 278CRL
- Disposable boot covers - Arnold David Cohen 244
- Disposable vinyl gloves
- Disposable hats
- Adhesive entrance mats - Arnold David Cohen A6125-20W
- Kimwipes
- Wescodyne

B. Incubator maintenance

- Fyrite gas analyzer - UWR Scientific 32033-000
- Standard sampling assembly - Metro Pak Ind. 11-7029
- Fyrite CO₂ fluid - Metro Pak Ind. 10-5057
- Carbon dioxide cylinders
- Incubator thermometer - Daigger Scientific 9325-H10

III. METHODS

A. Incubators

1. Incubator CO₂ monitoring (daily)

a. Fyrite meter initialization and readings

1. Wet interior of meter by inverting it then returning it to its upright position.

**ERL-N STANDARD OPERATING PROCEDURE
CLEAN ROOM MAINTENANCE**

**ERL-N SOP 1.01.001
REVISION 0
DECEMBER 1990
PAGE 2 OF 6**

2. Check the sampling assembly for cracks or leaking and connect to the hose located at the side of the incubator. The other end is pressed to the top of the fyrite meter, and the bulb on the sampling assembly is squeezed 18 times and removed from the meter.

3. The meter is inverted until fyrite fluid is finished flowing and the meter is returned to its normal position. The fyrite fluid then flows back down (CO₂ readings would be taken at this step).

4. The plunger at the top is released and the meter is zeroed by positioning the 0 on the ruler to line up with the top of the fyrite fluid.

b. CO₂ monitoring (fyrite readings)

1. Follow the procedure above, record fyrite reading and the digital incubator reading.

2. Temperature Monitoring (daily)

a. Open door of incubator without opening inner glass door and record the temperature of the incubator.

b. Record the incubator thermometer dial reading weekly and whether it is set to variable or not.

3. Humidity and Water Jacket (weekly)

a. Check water level at the bottom of incubator for water.

b. Whenever needed, sterile DI is added to inside of incubator. 2-3 liters of water is added once or twice every two weeks to the water jacket.

4. Incubator cleaning

a. Turn off the CO₂ valve behind the incubator before beginning.

b. Autoclave several liters of DI.

c. Remove all trays, thermometer and shelves from the incubator and have the trays and shelves autoclaved.

d. Vacuum water from the bottom of the incubator.

e. Spray the interior of the incubator with Wescodyne. Scrub and rinse with the hot autoclaved DI. Vacuum and repeat rinse to get rid of the Wescodyne.

f. Wipe glass door with 70% ethanol.

g. Return autoclaved shelves and trays, and sterilized thermometer to incubator.

- h. Refill water inside the incubator with sterile DI and turn the CO₂ back on.

B. Clean room

1. After cleaning any incubator(s), the floor and surfaces are mopped with diluted Wescodyne.
2. Pipettes and plates are stocked in drawers and cupboards.
3. Hoods. Interiors are wiped with Wescodyne prior to subculturing and testing.

C. Waterbath

1. Weekly: Add DI to the interior of the waterbath to maintain required depth so that test tubes can be thawed and bottles of media and components warmed to room temperature.
2. Monthly: Drain the waterbath, clean with diluted Wescodyne, add DI to required depth.

D. CO₂ cylinders (One tank runs and two tanks are in reserve)

1. Cylinder pressure is checked periodically and reserve tanks are used when pressure gets low.
2. When two cylinders become empty, they are removed to the chemical building and replaced with two full cylinders.
3. Check for leaks after replacing CO₂ tanks.

E. Liquid Nitrogen Storage tank

1. Once or twice a week LN₂ level in the storage tank is recorded using a wooden yardstick inserted along the neck until it reaches the bottom. The stick is withdrawn and the LN₂ level is recorded. If the level falls below 6", then the Dewar flask must be refilled from the main tank.

IV. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable to this SOP.

**ERL-N STANDARD OPERATING PROCEDURE
CLEAN ROOM MAINTENANCE**

**ERL-N SOP 1.01.001
REVISION 0
DECEMBER 1990
PAGE 4 OF 6**

V. TROUBLESHOOTING

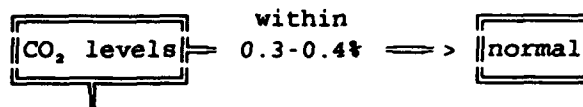
A. Incubators

1. CO₂ reading of digital meter on the incubator must be within 0.3-0.4% of the fyrite meter reading. All possible causes, as illustrated in the following flow chart should be examined before any adjustments are made to the CO₂ meter.

ERL-N STANDARD OPERATING PROCEDURE
CLEAN ROOM MAINTENANCE

ERL-N SOP 1.01.001
REVISION 0
DECEMBER 1990
PAGE 5 OF 6

Flow Chart for Incubator Readings Which Do Not Agree with Fyrite Readings



±0.4% for
more than
2 consecutive
days.

do various checks on
incubators, all must be
normal before proceeding.

TEMPERATURE --> DI-level JACKET --> DI-level CHAMBER --> FAN UNIT --> CO₂ TANKS
37±0.3°C full covering bottom functioning full, no leaks

make necessary corrections
to the above.

check fyrite readings after
incubators have stabilized. — within 0.3-0.4% —> normal

more than ±0.4%

do checks on fyrite meter

APPARATUS --> FYRITE FLUID
no leaks in >2 months old
hoses

normal

adjust CO₂ digital
meter to match fyrites

±0.4%

redo fyrite readings after
correcting the apparatus

within
0.3-0.4%

normal

within
0.3-0.4%

normal

**ERL-N STANDARD OPERATING PROCEDURE
CLEAN ROOM MAINTENANCE**

**ERL-N SOP 1.01.001
REVISION 0
DECEMBER 1990
PAGE 6 OF 6**

VI. REFERENCES
None.

**ERL-N STANDARD OPERATING PROCEDURE
HISTOLOGICAL PREPARATION FOR SHELLFISH
AND FISH**

**ERL-N SOP 1.01.002
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PAGE 1 OF 5**

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Narragansett, RI 02882**

I. OBJECTIVE

Histological preparation is the fixation and trimming of tissues to allow light-microscopic examination. The examination can be used to determine the presence or absence of pathological changes and evaluate the health of the animal or its exposure to contaminated material or infectious agents. Changes include morphological alterations, variations in the normal staining characteristics or a change in the rate of occurrence of features (i.e. mitotic figures). Techniques currently used are derived from the work of Yevich and Barszcz (1981) with a few modifications.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Fixation and initial trimming

- Fixative for fish (Dietrich's fixative):

- 9000 ml distilled water
- 4500 ml 95 % ethyl alcohol
- 1500 ml 40% formaldehyde
- 300 ml glacial acetic acid

Combine chemicals in the sequence listed, starting with water.

- Fixative for shellfish (Helly's fixative):

- 1000 gm zinc chloride
- 500 gm potassium dichromate
- 20 L distilled water

Stir with a magnetic stirrer until the chemicals are in solution. Add formaldehyde (unbuffered) at the time of fixation, using the following proportion: 1 ml of 37-40% formaldehyde for 20 ml of Helly's fixative. Once the formaldehyde has been added, Helly's has a shelf life of 24 hours. This is a modification of technique originally using mercuric chloride (Jones, 1974), now substituted with zinc chloride (Yevich and Barszcz, 1981). Note: other fixatives may be required if special stains are to be used. For electron microscopy, glutaraldehyde and osmium tetroxide or 10% buffered formalin are required.

**ERL-N STANDARD OPERATING PROCEDURE
HISTOLOGICAL PREPARATION FOR SHELLFISH
AND FISH**

**ERL-N SOP 1.01.002
REVISION 0
DECEMBER 1990
PAGE 2 OF 5**

- Magnetic stirrer and stir bars
- Scale
- Carboys, graduated cylinders for preparing and storing fixative.
- Glass bottles capable of holding a volume of fixative 20 times greater than the volume of tissue.
- Fume hood
- Florist's shears for dissection of flatfish
- Forceps
- Cutting board
- Measuring board
- Index cards, permanent felt-tip markers for labelling

B. Final trimming

- Disposable surgical blades
- Cassettes (stainless steel)
- Paper labels for cassettes
- No. 1 pencils (soft) for labelling
- Cutting board
- Gloves or finger cots
- Stainless steel cans to hold cassettes prior to processing - can also use large wide-mouth glass bottles but the possibility of breakage and spill make it less desirable than stainless steel.

C. Decalcification

- Decalcifying solution (20 L)
 - EDTA tetrasodium, 14.0 g
 - Sodium potassium tartrate, 0.16 g
 - Sodium tartrate, 2.8 g
 - Hydrochloric acid, 1984.0 ml
 - Deionized water

Bring to total volume of 20 liters with deionized water.

- Fume hood
- Safety equipment including acid-resistant gloves
- Carboy, glass graduated cylinder.
- Scale

D. Tissue washing

- Running tap water
- Tissue washer which alternately fills and discharges water over the cassettes.
- 1 cm-mesh bags

III. METHODS

A. Test Animals:

Bivalves may either be purchased from culture facilities or collected from the field.

B. Tissue extraction of shellfish

1. Insert a shellfish knife between the valves being careful not to damage the tissue.
2. Separate the valves by turning the knife blade sideways.
3. Gently slide the knife along the dorsal valve to detach the mantle and adductor muscle; remove the shell.
4. Detach the adductor muscle from the ventral valve using the same technique.
5. Examine the tissue grossly for parasites, cysts or tumors or other changes in color or shape.
6. Immerse the tissue in fixative until firm (generally 15-30 min.).
7. When firm, sagittally section leaving the halves attached at one end. With unusually large animals, parasagittal cuts may be necessary to allow proper fixation. Proper cutting uses firm pressure with a slow sawing motion. Switch to a new blade as the old one dulls to assure a smooth cut with minimum disruption of tissue.
8. Return the tissue to the fixative for no more than 16 hours. Overfixation in Helly's fixative increases the fragility of the section.
9. For final trimming, slice transversely through the full length of the body near the pericardial cavity.
10. Cut each half parasagittally into 2-3 mm-thick sections and approximately 2.5 cm in diameter to fit into cassettes and embedding molds. Do not force sections to fit into cassettes. Place section into cassette with the side to be cut facing down. Include an identification tag in each cassette. Generally, there are at least four tissue blocks for each bivalve.
11. Wash at least 16 hours in a siphoning water bath. Transfer to 70% ethyl alcohol.

C. Tissue extraction from fish

1. Fish less than 7.5 cm are immersed live in Dietrich's fixative. After they are dead, make an incision from the mouth to the anus almost completely through the body. Reimmerse in fixative for at least 8 hours. Decalcify.
2. Fish between 7.5 and 25 cm. are immersed live in Dietrich's fixative. After they are dead, make an incision in the abdominal cavity and remove internal organs.

**ERL-N STANDARD OPERATING PROCEDURE
HISTOLOGICAL PREPARATION FOR SHELLFISH
AND FISH**

ERL-N SOP 1.01.002
REVISION 0
DECEMBER 1990
PAGE 4 OF 5

3. Fish greater than 25 cm. can be killed prior to dissection. For dissection of flatfish, use florist's shears to start an incision just above the brain and lateral line through the width of the body. Continue until the cut has been made past the kidney and visceral cavity. Turn and cut through the vertebral column and around the visceral cavity until the muscle or fillet of the fish has been separated from the major organs.

4. Using forceps and a surgical blade, tease the liver and GI tract from the cavity and immerse in fixative. The liver should be attached in two places. By gently lifting the liver with the forceps, the anterior and posterior connections will become evident. Cut the head and gills from the body. Make sure that the brain area is exposed. Immerse in fixative. Gonadal tissue, lying posteriorly to the visceral cavity, should also be removed and immersed in fixative. Cut the kidney from the visceral cavity, leaving the vertebrae attached to the kidney. Make an incision in the center of the kidney and immerse in fixative.

5. When the tissue has become firm (15-30 minutes), remove the liver and GI tract. Make several parasagittal cuts in the liver. The number of cuts needed is dependent on the thickness of the liver tissue. Tissue should be no thicker than 4 mm. Next, remove the spleen from within the loop of the GI tract; if the spleen is sufficiently large (thicker than 4 mm) make an incision almost through the spleen and reimmerse in fixative. Slice open the gastrointestinal tract, and, using forceps, remove debris. Reimmerse in fixative.

IV. TROUBLE SHOOTING

A. Fixation and Trimming

1. If the tissue is brittle and falls apart when sectioning, it was probably left in fixative for too long.
2. If there are areas that are not firm, but are still soft and jelly-like, they probably have not fixed. Check the thickness of the tissue and if it is too thick, section again but note poor fixation in log book.

B. Decalcification

1. Surgical blades will dull very quickly when cutting tissue that has been decalcified. However, with a new blade, if it is still very difficult to cut through bone, the tissue needs more time to decalcify.

C. Tissue washing

1. If the alcohol bath after washing turns orange after having used Helly's fixative, there is still fixative left in the tissue. It must be removed before the tissue is processed. Make changes of the alcohol until very little fixative is leached out.

V. STATISTICAL ANALYSIS AND DATA USAGE

Microscopic evaluations at the cellular level are conducted on all organs but emphasize the kidney, gills, gonads, heart and digestive diverticula in shellfish and kidney, gills, liver, spleen and gastrointestinal tract in fish. Respiratory, digestive and excretory organs have potential to have direct exposure to toxic chemicals found in marine sediments.

Mathematical analysis is limited to a comparison of percent prevalence of a pathological change.

Quantitative analyses have been performed using areas of pathological change measured on an image analyzer.

VII. References

- Jones, R.M. 1964. McClung's handbook of microscopial technique. 3rd Edition. Hafner Publishing Co., New York.
- Yevich, P.P. and C.A. Barszcz. 1981. Preparation of aquatic animals for histopathological examination. DN 0543A, Aquatic Biol. Sect., Biol. Meth. Branch, Environmental Monitoring and Support Laboratory, U.S. EPA, Cincinnati, OH, 38 pp.

ERL-N STANDARD OPERATING PROCEDURE
CULTURING *Cyprinodon variegatus*, *Menidia beryllina*,
Mysidopsis bahia*, AND *Arbacia punctulata

ERL-N SOP 1.01.003
REVISION 0
JANUARY 1991
PAGE 1 OF 13

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I. OBJECTIVE

This standard operating procedure describes methods for culturing the fish species *Cyprinodon variegatus*, *Menidia beryllina*, the mysid *Mysidopsis bahia*, and the sea urchin *Arbacia punctulata* in the laboratory. Methods for culturing food organisms for these species are also included.

II. NECESSARY MATERIALS AND EQUIPMENT

A. General

- Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.
- Air pump -- for oil free air supply.
- Air lines, plastic or pasteur pipettes, or air stones.
- pH and DO (non-stirring probe) meters -- for routine physical and chemical measurements.
- Light box -- for counting and observing embryos and juvenile culture animals.
- Refractometer -- for determining salinity.
- Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.
- Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.
- Mini-Winkler bottles -- for dissolved oxygen calibrations.
- Wash bottles -- for deionized water and seawater.

- Tape, colored -- for labelling cultures and embryo dishes
- Markers, water-proof -- for marking containers, etc.
- Large supply of filtered and/or autoclaved seawater -- for culture water.
- Polyethylene wide-mouth jars (1 L) with screw-cap lids -- for shipping mysids and fish embryos/larvae.
- Soap and hot water -- for cleaning culture vessels.

ADDITIONAL EQUIPMENT:

A. Rotifer Culture

- Pyrex carboys with a drain spigot near the bottom, four 12 L capacity -- to hold cultures.
- Temperature-controlled room or chamber (25-28°C) -- for maintenance of cultures.
- Start-up sample of rotifers.
- NITEX[®] screen, < 80 μ m -- to harvest rotifers.
- Formalin -- to kill rotifers for counting.
- Sedgwick-Rafter counting chamber -- for counting rotifers.
- Large (4-5 L) plastic beakers -- to hold algae for feeding.

B. Algal Culture

- Start-up cultures of each algal species -- available from algal group.
- Polycarbonate carboys, 20 L -- to hold algal cultures.
- Temperature-controlled room or chamber (18-20°C) -- for maintenance of cultures.
- NaNO₃.
- NaH₂PO₄.
- EDTA.
- FeCl₃ · 6H₂O.
- Foam stoppers.
- Magnetic stirrer and stir bars -- for dissolving nutrient solutions.
- Pyrex carboy, 12 L -- for mixing and holding nutrients.

C. Brine Shrimp (*Artemia*) Culture

- Brine shrimp cysts.
- Separatory funnels, 2 and 6 L -- for holding cultures.
- Temperature-controlled room or chamber (25°C) -- for maintenance of cultures.
- Graduated cuvette, 50 cc -- for measuring amount of cysts to add to funnel.
- Dark cloth or paper towels -- for darkening funnel.
- NITEX[®] screen, 150 μ m -- for harvesting nauplii.
- Large (100 L) tub -- for enrichment of *Artemia* for mysids.
- Commercial enrichment product -- for enrichment of *Artemia* for mysids.
- Top-loader balance -- for weighing enrichment product.

D. *Cyprinodon variegatus* Culture

- Temperature-controlled room or chamber (27°C) -- for maintenance of cultures.

ERL-N STANDARD OPERATING PROCEDURE
CULTURING *Cyprinodon variegatus*, *Menidia beryllina*,
Mysidopsis bahia, AND *Arbacia punctulata*

ERL-N SOP 1.01.003
REVISION 0
JANUARY 1991
PAGE 3 OF 13

- Adult sheepshead minnows -- for use as brood stock.
- Beach seine (3 - 6 mm mesh) -- for field collecting adults.
- Large (30 gal.) garbage can -- for transport of field-collected adults to the laboratory.
- Newly-hatched Reference *Artemia* nauplii -- for feeding larval and juvenile fish used in tests.
- Flake food such as TETRA Standard Mix SM-80^R -- for feeding adult fish.
- Temperature-controlled room or chamber (18-20°C, 25°C) -- for maintaining adult fish.
- Flow-through or recirculating, aerated tank system (all glass aquarium or "Living Stream" -- for maintaining adult fish.
- Undergravel filter system -- for maintaining water quality in culture tanks.
- Squirt bottles containing seawater -- for washing embryos.
- Spawning aquaria, 37 L -- for spawning adults.
- Rectangular filter material -- for use as spawning substrate.
- Large (~8 inch diameter) crystallization dishes -- for holding embryos.
- Large (~9-10 inch diameter) watch glasses -- for covering crystallization dishes.

E. *Menidia beryllina* Culture

- *Menidia beryllina* adults -- for use as brood stock.
- Beach seine (3 - 6 mm mesh) -- for field collecting adults.
- Bucket or large beaker -- for removing adults from beach seine.
- Large (30 gal.) plastic garbage can -- for transporting field-collected adults to the laboratory.
- Tank, at least 0.9 m in diameter, with flowing filtered seawater -- for holding adults.
- Temperature-controlled room or chamber (25°C) -- for maintaining cultures.
- Flake food such as TETRA Standard Mix SM-80^R -- for feeding adult fish.
- *Artemia* nauplii, live -- for feeding cultures.
- Siphon -- for cleaning the detritus from the bottom of tanks.
- Polyester aquarium filter-fiber -- for spawning substrate.
- String and weight -- for suspending filter-fiber in tank.
- Aquaria, 10 gal. -- for holding floss and larval fish.
- Rotifers -- for feeding early larvae.
- Newly hatched (less than 12-h old) Reference *Artemia* nauplii -- for feeding fish larvae prior to testing.

F. *Mysidopsis bahia* Culture

- Glass aquaria (120- to 200-L) -- for holding adult mysids.
- Undergravel filters -- to provide aeration and a current conducive to feeding.
- Prewashed, coarse (2-5 mm) dolomite substrate -- to cover undergravel filter.
- Newly-hatched and/or enriched *Artemia* nauplii -- to condition tank and feed cultures.
- Adult mysids -- for use as brood stock.
- Temperature-controlled room or chamber (25 - 27°C) -- for maintaining cultures.
- Clear glass or plastic panel -- to cover cultures.
- Bleach -- for cleaning tanks.
- Wide bore (4mm) pipette and bulb -- for transferring mysids.
- Heated filtered seawater -- for culture water.

- Aquarium nets -- for transferring mysids.
- Finger bowls, 20 cm diameter -- for holding mysids.
- Netted (1000 um) flow-through containers -- for holding gravid mysids (Figure 1).
- Glass wide-mouth separatory funnels, 4 L -- for holding gravid mysids (Figure 1).
- Glass aquaria (20 L) -- for holding juvenile mysids.

G. *Arbacia punctulata* Culture

- Adult sea urchins (*Arbacia punctulata*) -- for culture animals.
- Transformer, 10 - 12 V with electrodes -- for spawning adult urchins.
- Fiberglass tanks, 20 L -- for holding cultures.
- Temperature-controlled chamber ($15^{\circ}\text{C} \pm 3^{\circ}\text{C}$) or water refrigeration device -- for maintaining proper water temperature in cultures.
- Kelp (*Laminaria sp.*) or romaine lettuce -- for feeding adult urchins.
- Insulated boxes or coolers -- for shipping adult sea urchins.
- 40-L glass aquarium or INSTANT OCEAN[®] aquarium -- for holding animals outside the laboratory.
- Gravel bed filtration system -- for maintaining water quality in temporary holding facilities.

III. METHODS

A. Rotifer Culture

At hatching *Menidia beryllina* larvae are too small to ingest *Artemia* and must be fed rotifers (*Brachionus plicatilis*). The rotifers can be maintained in continuous culture when fed algae. Four 12 L culture carboys should be maintained simultaneously to optimize production.

1. Fill clean 12 L Pyrex carboys (with a drain spigot near the bottom) with cooled autoclaved Narragansett Bay seawater.
2. When the sea water has reached $25-28^{\circ}\text{C}$, aerate the water and add a start-up sample of rotifers (50 rotifers/mL) and food (about 1 L of dense *Tetraselmus suecica* culture (see Section B)).
3. The carboys should be checked daily to ensure that adequate food is available and that the rotifer density is adequate.
 - a. If the water appears clear, drain 2 L of culture water and replace it with algae. Excess water can be removed through the spigot drain and filtered through a < 80 um mesh screen. Rotifers collected on the screen should be returned to the culture.
 - b. If a precise measure of the rotifer population is needed, rotifers collected from a known volume of water can be resuspended in a smaller volume, killed with formalin and counted in a Sedgwick-Rafter cell. If the density exceeds 50 rotifers/mL, the amount of food per day should be increased to 2 L of algae

ERL-N STANDARD OPERATING PROCEDURE
CULTURING *Cyprinodon variegatus*, *Menidia beryllina*,
Mysidopsis bahia, AND *Arbacia punctulata*

ERL-N SOP 1.01.003
REVISION 0
JANUARY 1991
PAGE 5 OF 13

suspension.

4. The optimum density of approximately 200 rotifers/mL may be reached in 7 - 10 days and is sustainable for 2 months. At these densities, the rotifers should be cropped daily. Keeping the carboys away from light will reduce the amount of attached algae on the carboy walls. When detritus accumulates, populations of ciliates, nematodes, or harpacticoid copepods that may have been inadvertently introduced can rapidly take over the culture. If this occurs, discard the cultures.

B. Algal Culture (*Tetraselmus suecica*)

1. Filtered sea water is added to clean 20-L polycarbonate carboys (normally used for bottled drinking water) and then autoclaved (110°C for 30 min).

2. After cooling to room temperature, the carboys are placed in a controlled temperature chamber at 18-20°C.

3. One liter of algal starter culture (available from macroalgae group) and 100 mL of nutrients are added to one carboy. Formula for algal culture nutrients:

a. Add 180 g NaNO_3 , 12 g NaH_2PO_4 , and 6.16 g EDTA to 12 L of deionized water. Mix with a magnetic stirrer until all salts are dissolved (at least 1 h).

b. Add 3.78 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and stir again. The solution should be bright yellow.

4. The algal culture is vigorously aerated via a pipette inserted through a foam stopper at the top of the carboy. A dense algal culture should develop in 7 - 10 days and should be used by Day 21.

5. Maintain the original culture carboy as a source of 1 L aliquots of algae to start other carboys (always adding 100 mL of nutrients to each). Use the secondary carboys to feed the rotifers. Start-up of cultures should be made on a daily or every second day basis. Approximately 6 - 8 continuous cultures will meet the feeding requirements of four 12-L rotifer cultures.

6. When emptied, carboys are washed with soap and water and rinsed thoroughly with deionized water before reuse.

C. Brine Shrimp (*Artemia*) Culture

All *C. variegatus*, *M. beryllina*, and *M. bahia* larvae and juveniles used in ERL-N toxicity testing must be fed Reference *Artemia* only, prior to and during the test. *Artemia* used at ERL-N fall into two categories: those used for maintenance of brood stock cultures only and those used to feed the test organisms (including the period from hatching until the beginning of the test). *Artemia* in the first category are obtained from commercial suppliers, whereas those in the second category are exclusively EPA Reference *Artemia* Cysts (RAC). Currently RAC II is being used at ERL-N, but RAC III is available from the Quality

ERL-N STANDARD OPERATING PROCEDURE
CULTURING *Cyprinodon variegatus*, *Menidia beryllina*,
Mysidopsis bahia*, AND *Arbacia punctulata

ERL-N SOP 1.01.003
REVISION 0
JANUARY 1991
PAGE 6 OF 13

Assurance Research Division, EMSL-Cincinnati, OH (phone: FTS 684-7325). *Artemia* from Great Salt Lake, Utah, (GSL) are normally used for broodstock cultures. GSL nauplii are fed directly to brood stock fish, but are fed to mysid cultures after an enrichment procedure is applied (see below).

1. Add filtered seawater to a separatory funnel: 2 L sep. funnels are used for RAC hatching and 6 L funnels for GSL hatching.
2. Add 10 mL *Artemia* cysts per liter to the separatory funnel and aerate for at least 24 h at 25°C. (Hatching time varies with incubation temperature and the geographic strain of *Artemia* used).
3. To harvest, cut off the air supply in the separatory funnel. *Artemia* nauplii are phototactic. Therefore, place a dark cloth or paper towel over the top of the separatory funnel for 5-10 min, allowing the nauplii to concentrate at the bottom. Leaving the nauplii concentrated on the bottom much longer than 10 min without aeration will result in mortality.
4. Drain the nauplii onto a 150 um NITEX[®] screen and rinse with sea water before use.
5. Enrich *Artemia* nauplii in a 100 L volume of filtered sea water (density not to exceed 300,000 nauplii/L) by adding a commercial enrichment product (Selco[®] is currently being used at the rate of 0.3 g/L) and aerating vigorously for less than 24 h (preferably 15 h). Harvest enriched nauplii on a 150 um mesh screen and rinse 4-5 times with clean seawater before use.

D. *Cyprinodon variegatus* Culture

Sheepshead minnows can be continuously cultured in the laboratory from eggs to adults. Adult sheepshead minnows for use as brood stock may be obtained by seine in Atlantic coast estuaries, from commercial sources, or from young fish raised to maturity in the laboratory. Feral brood stocks and first generation laboratory fish are preferred to minimize inbreeding. Adults are maintained in natural seawater in a flow-through aerated system consisting of a glass or fiberglass aquarium or tank. The system is equipped with an undergravel biological filter and is maintained at ambient Narragansett Bay salinity and a photoperiod of 14 h light/10 h dark.

1. To detect disease and to allow time for acute mortality due to the stress of capture, field-caught adults are observed in the laboratory a minimum of two weeks before using as a source of gametes. Injured or diseased fish are discarded.
2. Cultures of adult fish to be used for spawning are maintained at 18-20°C until embryos are required.
3. Seven or eight days before larvae are required, transfer the adult fish (8-9 females and 2-3 males) to 37 L aquaria with undergravel filters in a 25°C room. The gravel in each aquarium is covered with 12 in. x 4 in. filter pads on which the fish will spawn.

ERL-N STANDARD OPERATING PROCEDURE
CULTURING *Cyprinodon variegatus*, *Menidia beryllina*,
Mysidopsis bahia*, AND *Arbacia punctulata

ERL-N SOP 1.01.003
REVISION 0
JANUARY 1991
PAGE 7 OF 13

4. After 24 - 48 hr, the filter pads are removed and the fish are returned to their 20°C tanks.
5. The pads are hung in filtered seawater (aerated) in a 37 L aquarium at 25°C.
6. About 3 days post-spawn, the embryos become pigmented and are easily seen on the filter pad. The embryos should be gently removed with a forceps and placed in a bowl of filtered seawater for further incubation in a static system.
 - a. Embryos should be aerated and incubated at 25°C, at a salinity of 20-30 ‰ and a 14-h photoperiod.
 - b. It is essential that daily water changes be provided to the developing embryos, and the dishes be covered to reduce evaporation that may cause increased salinity. One-half to three-quarters of the sea water from the culture vessels can be poured off and the incubating embryos retained.
7. Embryos cultured in this manner should hatch in six or seven days.
8. The larvae should be fed sufficient newly-hatched *Artemia* nauplii daily to assure that live nauplii are always present. Juveniles are fed a commercial flake food, such as TETRA Standard Mix. Adult fish (age one month) are fed flake food twice daily.
9. Sheepshead minnows reach sexual maturity in three-to-five months after hatch, and have an average standard length of approximately 27 mm for females and 34 mm for males. At this time, the males begin to exhibit sexual dimorphism and initiate territorial behavior. When the fish reach sexual maturity and are to be used for natural spawning, the temperature should be controlled at 18-20°C.

E. *Menidia beryllina* Culture

Menidia beryllina adults may be cultured in the laboratory or obtained from the Gulf of Mexico or Atlantic coast estuaries throughout the year. Gravid females can be collected from low salinity waters along the Atlantic coast during April to July, depending on the latitude. The most productive and protracted spawning stock can be obtained from adults brought into the laboratory. Broodstocks, collected from local estuaries twice each year (in April and October), will become sexually mature after 1-2 months and will generally spawn for 4-6 months.

1. The fish can be collected easily with a beach seine (3 - 6 mm mesh), but the seine should not be completely landed onto the beach. Silversides are very sensitive to handling and should never be removed from the water by net -- only by beaker or bucket.
2. Record the water temperature and salinity at each collection site.
3. Aerate the fish and transport to the laboratory as quickly as possible after collection.

4. Upon arrival at the laboratory, the fish and the water in which they were collected are transferred to a tank at least 0.9 m in diameter. A filter system should be employed to maintain water quality.
5. Laboratory water is added to the tank slowly, and the fish are acclimated at the rate of 2°C per day to a final temperature of 25°C and about 5 ‰ salinity per day to a final salinity in the range of 20-32 ‰. The seawater in each tank should be brought to a minimum volume of 150 L. A density of about 50 fish/tank is appropriate.
6. Maintain a photoperiod of 14 h light/10 h dark.
7. Feed the adult fish flake food twice daily and *Artemia* nauplii once daily.
8. Siphon the detritus from the bottom of the tanks weekly.
9. Fourteen days before larvae are required, stimulate the broodstock to spawn by suspending polyester aquarium filter-fiber (15 cm long X 10 cm wide X 10 cm thick) 8 - 10 cm below the surface of the water and in contact with the side of the holding tanks for 24 - 48 h. The floss should be gently aerated by placing it above an airstone, and weighted down with a heavy non-toxic object. The embryos, which are light yellow in color, can be seen on the floss, and are round and hard to the touch compared to the soft floss. The eggs are approximately 0.75 mm in diameter.
10. Remove as much floss as possible from the embryos. The floss should be stretched and teased to prevent the embryos from clumping.
11. The embryos should be incubated in a 37 L glass aquarium at the test salinity and lightly aerated. At 25°C, the embryos will hatch in about 6 - 7 days. Newly hatched larvae are 3.5-4.0 mm in total length.
12. Larvae should be fed rotifers ad libitum from hatch through four days post-hatch. On Days 5 and 6, newly hatched (less than 12-h old) RAC brine shrimp nauplii are mixed with the rotifers, to provide a transition period. After Day 7, only nauplii are fed, and the age range for the nauplii can be increased from 12-h old to 24-h old.
13. *Menidia* larvae are very sensitive to handling and shipping during the first week after hatching. For this reason, if organisms must be shipped to the test laboratory, it may be impractical to use larvae less than 11 days old because the sensitivity of younger organisms may result in excessive mortality during shipment. If organisms are to be shipped to a test site, they should be shipped only as (1) early embryos, so that they hatch after arrival, or (2) after they are known to be feeding well on brine shrimp nauplii (8 - 10 days of age). Larvae shipped at 8 - 10 days of age would be 9 - 11 days old when the test is started. Larvae that are hatched and reared in the test laboratory can be used at seven days of age.

F. *Mysidopsis bahia* Culture

ERL-N STANDARD OPERATING PROCEDURE
CULTURING *Cyprinodon variegatus*, *Menidia beryllina*,
Mysidopsis bahia*, AND *Arbacia punctulata

ERL-N SOP 1.01.003
REVISION 0
JANUARY 1991
PAGE 9 OF 13

The identification of the stock culture should be verified. Records of the verification should be retained along with a few preserved specimens.

1. Culture Conditions

- a. Glass aquaria (75 L) with flow-through sea water are used for cultures. About 30 aquaria are normally in operation to insure that a sufficient supply of juvenile mysids is always available.
- b. Standard aquarium undergravel filters should be used to provide aeration and a current conducive to feeding. The undergravel filter is covered with a prewashed, coarse (2-5 mm) dolomite substrate, 2.5 cm deep for flow-through cultures or 10 cm deep for recirculating cultures.
- c. It is important to add enough food to keep the adult animals from cannibalizing the young. However, in flow-through systems, excess food can be a problem by promoting bacterial growth. Just enough newly-hatched *Artemia* nauplii are fed twice a day so that each feeding is consumed before the next feeding. If no nauplii are present in the culture chamber after four hours, the amount of food should be increased slightly.
- d. *Mysidopsis bahia* should be cultured at a temperature of $25 \pm 2^{\circ}\text{C}$ and at ambient Narragansett Bay salinity. A 16-h/8-h day/night cycle in which the light is gradually increased and decreased to simulate dawn and dusk conditions, is recommended.
- e. Mysids cannot survive dissolved oxygen concentrations below 5 mg/L for extended periods. The airlift used in most undergravel filters will usually provide sufficient DO. If the DO drops below 60% saturation (4.8 mg/L at 25°C and 30 ppt salinity), additional aeration should be provided.
- f. Suspend a clear glass or plastic panel over the cultures, or use some other means of excluding dust and dirt, but leave enough space between the covers and culture tanks to allow circulation of air over the cultures.
- g. If hydroids or worms appear in the cultures, remove the mysids and clean the chambers thoroughly, using soap and hot water. Rinse once with acid (10% HCl) and three times with distilled or deionized water. Mysids with attached hydroids should be discarded. Those without hydroids should be transferred by hand pipetting into three changes of clean seawater before returning them to the cleaned culture chamber. To guard against predators, natural sea water should be filtered to 30 μm before entering the culture vessels.
- h. *Mysidopsis bahia* are very sensitive to low pH and sudden changes in temperature. Care should be taken to maintain the pH at 8.0 ± 0.3 , and to limit rapid changes in water temperature to less than 3°C .
- i. Mysids should be handled carefully and as little as possible so that they are not

unnecessarily stressed or injured. They should be transferred between culture chambers with long handled cups with netted bottoms. Animals should be transferred to the test vessels with a large bore pipette (4-mm), taking care to release the animals under the surface of the water. Discard any mysids that are injured during handling.

j. Careful culture maintenance is essential. The organisms should not be allowed to become too crowded. The cultures should be cropped as often as necessary to maintain a density of about 20 mysids per liter with a ratio of about 2 female mysids per male. At this density, at least 70% of the females should have eggs in their brood pouch. If they do not, the cultures are probably under stress, and the cause should be found and corrected. If the cause cannot be found, it may be necessary to re-start the cultures with a clean culture chamber, a new batch of culture water, and clean gravel.

k. It is recommended that the sides of the culture vessels be scraped and the gravel carefully turned over to prevent excessive build up of algal growth. Twice a year the mysids should be removed from the cultures, the gravel rinsed in clean seawater, the sides of the chamber washed with clean seawater, and the gravel and animals returned to the culture vessel with new sea water.

2. Obtaining Juvenile Mysids

Mysid tests are begun with 7-day old juveniles. To have the test animals available and acclimated to test conditions at the start of the test, they must be obtained from the stock culture eight days in advance of the test. Whenever possible, brood stock should be obtained from cultures having similar salinity, temperature, light regime, etc., as are to be used in the toxicity test.

a. Eight days before the test is to start, sufficient gravid females are placed in netted (1000 um) flow-through containers (brood chambers, Figure 1) held within 4-L glass, wide-mouth separatory funnels. When the cultures are in good condition, the number of juveniles obtained from each brood chamber is about 2 - 2.5 times the number of gravid females placed in the chamber.

1. Mysids are removed from the culture tank with a net or netted cup and placed in 20-cm diameter finger bowls.

2. The gravid females are transferred from the finger bowls to the brood chambers with a large-bore pipette or, alternatively, are transferred by pouring the contents of the finger bowls into the water in the brood chambers.

b. The temperature in the brood chamber should be maintained at the upper acceptable culture limit (26 - 27°C), or 1°C higher than the cultures, to encourage faster brood release.

c. The gravid females are fed newly hatched RAC nauplii, and are held

overnight to permit the release of young. The brood chambers usually require aeration to maintain sufficient DO and to keep food in suspension. Newly released juveniles can pass through the netting, whereas the females are retained.

d. The juvenile mysids are collected by opening the stopcock on the funnel and collecting them in another container from which they are transferred to holding tanks using a wide bore (4 mm ID) pipette.

e. The newly released juveniles (age = 0 days) are transferred to 20-L glass aquaria (holding vessels) which are gently aerated. Smaller holding vessels may be used, but the density of organisms should not exceed 20 mysids per liter. The test animals are held in the holding vessel for six days prior to initiation of the test. The holding medium is renewed every other day.

f. During the holding period, the mysids are acclimated to the salinity at which the test will be conducted, unless already at that salinity. The salinity should be changed no more than 2 ‰ per 24 h to minimize stress on the juveniles.

g. The temperature during the holding period is critical to mysid development, and must be maintained at 26 - 27°C. If the temperature cannot be maintained in this range, it is advisable to hold the juveniles an additional day before beginning the test.

h. During the holding period, just enough newly-hatched RAC nauplii are fed once a day (a total of at least 150 nauplii per mysid per day) so that some food is constantly present.

i. If the test is to be performed in the field, the juvenile mysids should be gently siphoned into 1-L polyethylene wide-mouth jars with screw-cap lids filled two-thirds full with clean seawater from the holding tank. The water in these jars is aerated for 10 min, and the jars are capped and packed in insulated boxes for shipment to the test site. Food should not be added to the jars to prevent the development of excessive bacterial growth and a reduction in DO.

j. Upon arrival at the test site (in less than 24 h) the mysids are gently poured from the jars into 20-cm diameter glass culture dishes. The jars are rinsed with salt water to dislodge any mysids that may adhere to the sides. If the water appears milky, siphon off half of it with a netted funnel (to avoid siphoning the mysids) and replace with clean salt water of the same salinity and temperature. If no *Artemia* nauplii are present in the dishes, feed about 150 nauplii per mysid.

G. *Arbacia punctulata* Culture

1. Adult sea urchins (*Arbacia punctulata*) can be obtained from commercial suppliers. After acquisition, the animals are sexed by briefly stimulating them with current from a 12 V transformer. Electrical stimulation causes the immediate release of masses of gametes that are readily identifiable by color -- the eggs are red, and the sperm are white.

2. The sexes are separated and maintained in 20-L, aerated fiberglass tanks, each holding about 20 adults. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or salt water prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded.
3. The culture unit should be maintained at $15^{\circ}\text{C} \pm 3^{\circ}\text{C}$, with a water temperature control device.
4. The food consists of kelp (*Laminaria sp.*), gathered from known uncontaminated zones or obtained from commercial supply houses whose kelp comes from known uncontaminated areas, or romaine lettuce. Fresh food is introduced into the tanks at approximately one week intervals. Decaying food is removed as necessary. Ample supplies of food should always be available to the sea urchins.
5. Natural seawater with a salinity of 30‰ is used to maintain the adult animals.
6. Adult male and female animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, aquaria (or a single partitioned aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to 15°C before animals are added. Healthy animals will attach to the kelp or aquarium within hours.
7. To successfully maintain about 25 adult animals for seven days at a field site, a screen-partitioned, 40-L glass aquarium using aerated, recirculating, clean saline water (30‰) and a gravel bed filtration system, is housed within a water bath, such as an INSTANT OCEAN[®] Aquarium (15°C). The inner aquarium is used to avoid contact of animals and water bath with cooling coils.

IV. TROUBLE SHOOTING

Included in Methods Section.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

- US EPA. 1988. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. Weber, C.I., et al (eds). EPA Office of Research and Development EPA-600/4-87/028 (May 1988).

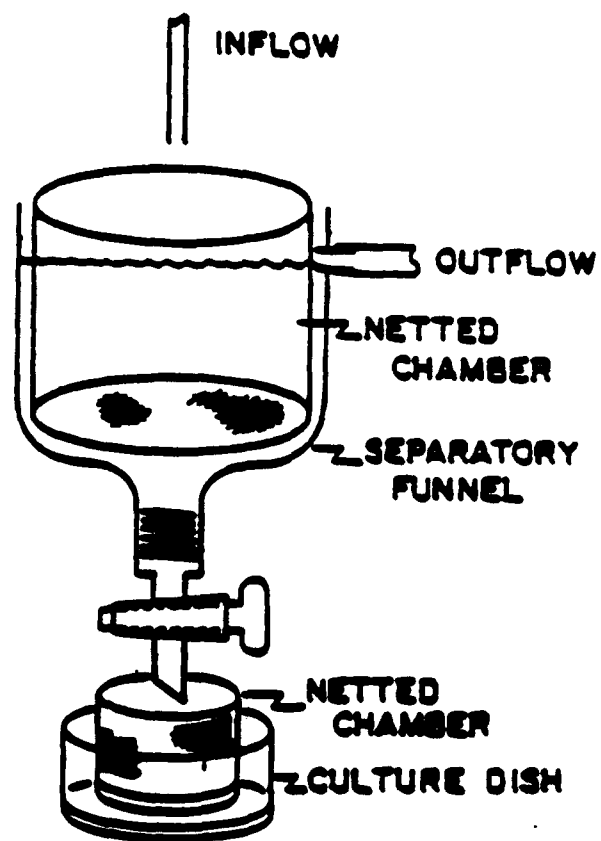


Figure 2. Apparatus (brood chamber) for collection of juvenile mysids. From Lussier, Kuhn, and Sewall, 1987.

**ERL-N STANDARD OPERATING PROCEDURE
PREPARATION OF HYPERSALINE BRINE FROM
NATURAL SEAWATER**

**ERL-N SOP 1.01.004
REVISION 0
DECEMBER 1990
PAGE 1 OF 3**

POINT OF CONTACT:

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I. OBJECTIVE

This method describes the preparation of hypersaline brine (HSB) for use in toxicity tests of surface water or effluent samples requiring salinity enhancement.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Brine Generation

- Container for making HSB--the ideal container for making brine from natural seawater is one that (1) has a high surface-to-volume ratio, (2) is made of a non-corrosive material, and (3) is easily cleaned (fiberglass containers are ideal).
- Uncontaminated source of natural seawater.
- Heat source--used to accelerate evaporation of brine.
- Aerator--used to accelerate evaporation of brine.
- Portable containers for storage of brine--carboys, cubitainers, or equivalent.
- Teflon-lined pump--for collecting brine and dispensing into storage containers.
- Filter apparatus with 10um filter for incoming seawater and 1um filter for brine.
- Refractometer--for monitoring the salinity of seawater and brine.
- Thermometer--for monitoring the temperature of brine.

B. Brine Use

- Water purification unit--deionized water or equivalent.
- Graduated cylinders--assorted sizes, for measuring volumes of HSB, deionized water, and effluent or receiving water.
- Beakers--assorted sizes, for mixing HSB, deionized water, and effluent or receiving water.

III. METHODS

**ERL-N STANDARD OPERATING PROCEDURE
PREPARATION OF HYPERSALINE BRINE FROM
NATURAL SEAWATER**

**ERL-N SOP 1.01.004
REVISION 0
DECEMBER 1990
PAGE 2 OF 3**

A. Brine Generation

1. Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough deionized water rinses.
2. High quality (and preferably high salinity) seawater should be filtered to at least 10 μ m before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.
3. The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.
4. After the desired salinity is attained (100‰), the brine should be filtered a second time through a 1 μ m filter and poured directly into portable containers, such as 20-L (5 gal) cubitainers or polycarbonate water cooler jugs. The containers should be capped and labelled with the date the brine was generated and its salinity. Containers of brine should be stored in the dark and maintained at room temperature until used. When stored in this manner, HSB may be held for prolonged periods without apparent degradation.
5. If a source of hypersaline brine is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and brine before adding the effluent.

B. Brine Use

1. Divide the salinity of the hypersaline brine by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 20‰, $100‰ \div 20‰ = 5.0$. The proportion of brine is 1 part in 5 (one part brine to four parts deionized water).
2. To make 1 L of seawater at 20‰ salinity from a hypersaline brine of 100‰, divide 1 L (1000 mL) by 5.0. The result, 200 mL, is the quantity of brine needed to make 1 L of seawater. The difference, 800 mL, is the quantity of deionized water required.

IV. TROUBLE SHOOTING

Special care should be used to prevent any toxic materials from coming in contact with

**ERL-N STANDARD OPERATING PROCEDURE
PREPARATION OF HYPERSALINE BRINE FROM
NATURAL SEAWATER**

**ERL N SOP 1.01.004
REVISION 0
DECEMBER 1990
PAGE 3 OF 3**

the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is used, use only oil-free air compressors to prevent contamination.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

U.S. Environmental Protection Agency. 1988. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. EPA Office of Research and Development EPA/600/4-87/028 (May 1988).

**ERL-N STANDARD OPERATING PROCEDURE
SEDIMENT GRAIN SIZE ANALYSIS**

**ERL-N SOP 1.01.005
REVISION 1
JULY 1991
PAGE 1 OF 5**

POINT OF CONTACT:

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I. OBJECTIVE

To determine the percentages by weight of sand, silt, and clay in sediment samples.

II. NECESSARY MATERIALS AND EQUIPMENT

- 9-16 g sediment (50-100 ml material)
- Pre-weighed 250 ml beakers
- Source of deionized water
- 0.063 mm sieve, 5 inch diameter
- Evaporation dish, 8.5 inch diameter
- Scale capable of weighing to 0.0001 g
- Laboratory oven (100°C)
- 1 L graduated cylinder
- Dispersant (sodium hexametaphosphate)
- Glass stirring rod
- 20 ml pipette with depth marks at 10 cm and 20 cm from the tip
- Thermometer
- Stop watch

III. METHODS

1. Place between 50 and 100 ml of material (9-16 g less than 0.063 mm) in a 250 ml beaker and disaggregate in a solution of deionized water for approximately 24 hrs.
2. Wet-sieve the disaggregated sample through a 0.063 mm sieve using deionized water and collect the water and <0.063 mm particles in an underlying evaporation dish.
3. Using a squeeze bottle filled with deionized water, rinse the >0.063 mm material (i.e. sand) from the sieve screen to a separate pre-weighed beaker.
4. Dry the sand at approximately 100°C and weigh to the nearest 0.01 g.
5. Subtract the beaker weight from the beaker + sand weight to determine the weight of the sand fraction.
6. Transfer the <0.063 mm material (i.e. silt and clay) from the evaporating dish to

a 1 L graduated cylinder.

7. Add 10ml (0.0025 g) of dispersant from stock solution of sodium hexametaphosphate to the cylinder.

8. Fill the column with deionized water to the 1000 ml mark and thoroughly mix using a glass stirring rod.

9. Cover the graduated cylinder with a watch glass and store the solution for 12 hours to observe for flocculation. Flocculation can be recognized by a curdling and rapid settling of clumps of particles or by the presence of a thick soupy layer on the bottom of the cylinder passing abruptly into relatively clear water above. If flocculation occurs, treat the sample with more dispersant, if possible discard the sample and repeat steps 1-9 with smaller amounts of sample. If more dispersant is added, thoroughly mix the sample again and repeat step 9.

10. Thoroughly stir the solution and pipette out aliquots at specified times and depths. Withdrawal times are determined based on water temperature at the beginning of the analysis as measured by thermometer. (see Table 1). STET, room temperature should be recorded.

a. 20 seconds after stirring, withdraw a 20 ml sample at a depth of 20 cm from the 1000 ml mark on the cylinder and place in a beaker (silt+clay fraction). This procedure is independent of temperature.

b. Later, withdraw a second 20 ml sample at a depth of 5 cm from the water surface in the cylinder and place in a beaker (clay fraction). The timing of this procedure is temperature dependent and is determined by referral to Table 1.

11. Oven dry the material in the beakers at 100-110°C for approximately 24 hours.

12. Cool the materials in a weighing room for 2-3 hours to allow the clays to equilibrate with ambient humidity.

13. Weigh the beakers and contents to the nearest 0.0001 g. Subtract the weight of the beakers to determine the weight of the silt+clay and clay fractions.

14. Multiply the weight of the sediment (silt + clay fraction) by 50 and subtract the weight of one dispersant to obtain the total weight of silt + clay in the cylinder.

15. Multiply the weight of the clay fraction by 50 and subtract the weight of the dispersant to obtain the total weight of clay in the cylinder.

16. Subtract the weight of the clay fraction from the silt+clay fraction to obtain the weight of the silt fraction.

17. Add the weights of the sand, silt, and clay fractions together to obtain the total sample weight.

**ERL-N STANDARD OPERATING PROCEDURE
SEDIMENT GRAIN SIZE ANALYSIS**

**ERL-N SOP 1.01.005
REVISION 1
JULY 1991
PAGE 3 OF 5**

18. Determine the percentages of sand, silt, and clay by dividing the individual weights by the total sample weight and multiplying by 100.

IV. TROUBLE SHOOTING

Included in methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

Lewis, D.W. 1984. "Texture of Detrital Sediments". In Practical Sedimentology. Hutchinson Ross Pub. Co., Stroudsburg, PA, pg. 106.

Folk, R.L. 1968. Petrology of Sedimentary Rocks. Herphill Publishers, Austin, TX.

Sediment QA/QC

Quality control for the sediment analysis procedures will be accomplished by reanalyzing samples that fail either a Range Check or Recovery Check. Quality assessment will include reanalysis of 10% of the samples. Samples for which quality assessment are to be conducted will be selected randomly. Reanalysis will consist of repeating the sediment analysis procedure on the archived sample collected in the field from the same grab as the sample failing QC.

For the Range Check, any sample results that fall outside expected ranges will be reanalyzed. For example, any percentage that totals greater than 100% will be reanalyzed. For the Recovery Check, if the total weight of the recovered sample is 10% (by weight) less or greater than the starting weight of the sample, the sample will be reanalyzed.

For Quality Assessment, samples will be selected randomly from each batch and reanalyzed. A batch of samples is a set of samples of a single textural classification (ie., mud) processed by a single technician using a single procedure (ie., complete sediment analysis).

Sediment sample reanalysis will be done in the following manner:

1. Approximately 10% of each batch completed by the same technician will be reanalyzed.
2. A random selection of the samples will be processed in the same manner as the original sample batch.
3. If the absolute difference between the original sand-silt-clay percentage and the second sand-silt-clay percentage is greater than 10% then a third analysis will be completed by a different technician.
4. The values closest to the third value will be entered into the data base.
5. If more than 10% of the data from a batch are found in error, then the whole batch will be reprocessed using the archived sediment. A third check of the reanalyzed samples will be complete by a different technician to assure that the reanalyzed values are correct.
6. Reanalysis and QA checks must be accomplished within 30 days from the date the original sediment analysis was conducted.

TABLE 1.
SAMPLING TIME INTERVALS FOR PIPETTE ANALYSIS AT DIFFERING TEMPERATURES.

Phi Diameter	depth mm (cm)	18°C	19°C	20°C	21°C	22°C	23°C	24°C	25°C	26°C
4.0	0.062 20	:20	:20	:20	:20	:20	:20	:20	:20	:20
4.5	0.044 20	2:00	1:59	1:56	1:53	1:50	1:47	1:45	1:43	1:41
5.0	0.031 10	2:00	1:59	1:57	1:53	1:51	1:49	1:46	1:44	1:41
5.5	0.022 10	4:03	3:58	3:52	3:46	3:40	3:35	3:30	3:26	3:21
6.0	0.015 10	8:43	8:31	8:12	8:05	7:54	7:43	7:32	7:22	7:13
6.5	0.011 10	16:12	15:50	15:27	15:03	14:41	14:21	14:01	13:43	13:24
7.0	0.0078 10	32:14	31:29	31:00	29:56	29:13	28:32	27:53	27:16	26:40
7.5	0.0055									
8.0	0.0039 5	1:04:00	1:03	1:01:00	59:53	58:26	57:04	55:46	54:31	53:19
8.5	0.0028									
9.0	0.0020 5	4:04:00	3:39	3:53:00	3:47	3:42:00	3:37:00	3:32:00	3:27	3:23:00

**ERL-N STANDARD OPERATING PROCEDURE
GENERAL DATA ENTRY**

**ERL-N SOP 1.01.006
REVISION 0
DECEMBER 1990
PAGE 1 OF 2**

POINT OF CONTACT:

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I. OBJECTIVE

The purpose of this SOP is to provide a guideline for general data entry and quality assurance procedures.

II. NECESSARY MATERIALS AND EQUIPMENT

- A. Original data sheets. These forms should be completely, accurately, clearly, and consistently filled out and QA'ed by qualified personnel.**
- B. Personal computer or mainframe with data management software and a printer.**

III. METHODS

- A. Data should be entered into a computer database (approved by computer staff if appropriate) completely, accurately, and consistently.**
- B. A hard copy of the information contained in the database should be printed out, and all of the data should be checked for accuracy against the original datasheets.**
- C. Corrections should be made to the data in the database as is necessary.**
- D. Corrected data should be re-printed and QA'd a second time.**
- E. Data should be distributed to the appropriate personnel for review or analysis.**

IV. TROUBLE SHOOTING

- A. Computer databases should be designed or approved by qualified computer experts prior to data entry in order to prevent loss of data.**
- B. At least two copies of the original datasheets should be kept on file in order to prevent loss of data.**

**ERL-N STANDARD OPERATING PROCEDURE
GENERAL DATA ENTRY**

**ERL-N SOP 1.01.006
REVISION 0
DECEMBER 1990
PAGE 2 OF 2**

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

Not applicable.

**ERL-N STANDARD OPERATING PROCEDURE
CELL SUBCULTURE METHODS**

**ERL-N SOP 1.01.007
REVISION 0
DECEMBER 1990
PAGE 1 OF 3**

POINT OF CONTACT:

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I. OBJECTIVE

This SOP describes methods required to maintain mammalian tissue culture cells (Chinese Hamster Lung Fibroblast) for experimental procedures (ie. Sister Chromatid Exchange Assay, Chromosomal Aberrations Assay, Micronucleus Assay, Metabolic Cooperation Assay, Toxicity Test).

II. MATERIALS AND EQUIPMENT

A. Media reagents. Note: Store as recommended by manufacturer.

- Minimum Essential Media (Gibco 320-1090)
- Earles Balanced Salt Solution (Gibco 310-4150)
- Essential Amino Acids (Gibco 320-1130)
- Non-essential Amino Acids (Gibco 320-1140)
- NaCl solution (8.35 g NaCl/100ml deionized water)
- L-glutamine (Gibco 320-5030)
- Vitamins (Gibco 320-1120)
- Fetal Bovine Serum (Gibco 200-6140)

B. Other materials and equipment.

- Sterile clean room garments, (hats, lab coats, booties, etc.) needed to maintain an aseptic environment. Worn in designated clean room area.
- Water bath for to warm frozen and refrigerated components.
- Automatic pipetters to facilitate manipulation of media, components, and cells.
- Sterile stripette pipettes (1 ml - CoStar 4015, 5 ml - CoStar 4055, 10 ml - CoStar 4105, 25 ml - CoStar 4251) for use with automatic pipetter.
- Horizontal laminar flow hood (Baker Co. G6252) to maintain a sterile environment.
- Wescodyne to sterilize clean room surfaces.
- 70% ethanol to disinfect media bottles, tubes and hands.
- Vacuum system to facilitate removal of spent media and rinses from plates.
- Sterile 5 3/4" Pasteur pipettes for use with vacuum (Daigger Scientific 7760-A12).
- Incubators for maintenance of cell cultures at adequate pH (5% CO₂), temperature (37°C) and humidity (saturated). Note: See SOP on Incubator and clean room maintenance.

**ERL-N STANDARD OPERATING PROCEDURE
CELL SUBCULTURE METHODS**

ERL-N SOP 1.01.007
REVISION 0
DECEMBER 1990
PAGE 2 OF 3

- Trypsin (Gibco 610-5090) to disassociate cells.
- Sterile 50 ml Corning centrifuge tubes (Daigger Scientific 25339-50) for cells suspension.
- Sterile 100 x 15mm NUNC plastic tissue culture plates (Daigger Scientific 150-350) for cell maintenance.

III. METHODS Note: All procedures must be conducted in a sterile environment using aseptic techniques.

A. Media Preparation.

Media/100ml.

10 ml Fetal bovine serum
1 ml L-glutamine
1 ml Non-essential Amino Acids
1 ml Essential Amino Acids
1 ml NaCl
0.5 ml Vitamins
85.5 ml Minimum Essential Media.

1. Bottles and tubes of components, and an empty sterilized bottle are rinsed and wiped with 70% ethanol prior to being placed into laminar flow hood.
2. Following the above growth media recipe, media components are measured in desired quantities into an empty, sterilized bottle.

B. Harvesting Procedure

1. Plates are examined on an inverted microscope for contamination and confluency and selected for testing or maintenance.
2. Old growth media is vacuumed into a liquid waste container using a sterile 5 3/4" pasteur pipette fitted on the end of silastic tubing that is connected to a vacuum unit.
3. The cultures are rinsed with 5 ml Earles Balanced Salt Solution and vacuumed, then repeated for a total of 2 rinses.
4. 0.5 ml trypsin is added to each plate and incubated for 5 minutes until cells begin to detach.
5. 5 ml of media is added to each plate to inhibit the trypsin.
6. Cells are removed from the plate by vigorous pipetting with an automatic pipetter. The detached cells and media are termed "cell suspension".

C. Seeding Procedure

**ERL-N STANDARD OPERATING PROCEDURE
CELL SUBCULTURE METHODS**

**ERL-N SOP 1.01.007
REVISION 0
DECEMBER 1990
PAGE 3 OF 3**

1. For a two day culture, 0.7ml of cell suspension is added per 25ml growth media.
2. For a three day culture, 0.3ml of cell suspension is added per 25ml growth media.

10ml of the above cell suspension and media is put into sterile 100x15 NUNC tissue culture plates and returned to the incubator.

IV. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable to this SOP.

V. TROUBLE SHOOTING

Contaminated plates are removed, secured with autoclave tape, wrapped in aluminum foil and autoclaved. Incubators are cleaned and sterilized before initiating new cultures which can be obtained from frozen cells maintained in liquid nitrogen.

VI. REFERENCES

Malcolm and Mills, modified media recipe.

**ERL-N STANDARD OPERATING PROCEDURE
BENTHIC ORGANISM COLLECTION**

**ERL-N SOP 1.02.001
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PAGE 1 OF 4**

POINT OF CONTACT:

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I. OBJECTIVE

Collect infaunal and epibenthic marine organisms for subsequent tissue residue analyses or for use in toxicological evaluation.

II. MATERIALS AND EQUIPMENT

The equipment needed and the method of collection will vary depending on the organism(s) to be collected. Certain general materials and equipment should be considered standard for all types of field collections.

General:

- Foul weather gear
- Boots
- Water proof notebook
- Water proof markers
- Twine knife
- Line and twine
- Appropriate clothing
- Marine radio
- Standard safety equipment
- First Aid kit
- Exposure suits (orange worksuits) for winter sampling
- Gloves - appropriate gloves for suspected conditions
- Compass - handheld for taking sitings at sample sites
- Electrical tape
- Duct Tape

A. Worms

- Sediment Grab - Smith-MacIntyre
- Stand - for the grab
- Lead weights - 4 triangular lead weights for Smith-Mac
- Hard hat and face shield
- Plywood pads for Smith-Mac, if soft sediments are anticipated
- Respirator - for highly contaminated areas
- Caulking bar for Smith-Mac
- Sediment scoops - teflon

**ERL-N STANDARD OPERATING PROCEDURE
BENTHIC ORGANISM COLLECTION**

ERL-N SOP 1.02.001
REVISION 0
FEBRUARY 1990
PAGE 2 OF 4

- Tub(s) - into which to dump sediment
- Sieves - 2, 1, and 0.5mm
- Sieving stand(s)
- Deck hose with spray nozzle
- Forceps - plastic
- Sample containers - jars (cleaned and acid stripped)
- Carolina bowls - rinsing and segregating
- Water proof labels
- Coolers with ice, or other means of cold storage
- Water-proof sample notebook
- Water-proof markers
- Buckets (2-3) - for rinsing
- Cable cutter
- Cable crimping tools
- Tools - screw drivers, wrenches, hammer, other

B. Quahogs

- Hire a professional quahog fisherman
- Mesh bags (one per sample site) (labelled)

C. Mussels

- 17' Whaler or larger vessel
- Scallop dredge
- Towing line (5:1 line length:depth)
- Plastic mussel baskets

D. Oysters

- Pocket knife (with locking blade or other prying device)
- Mesh bags (one per sample site) labelled

E. Soft-shell clams

- 17' Whaler
- Oars (for moving the boat in shallow waters)
- Clam fork, garden fork
- Waders
- Mesh bags

III. METHODS

A. Worms

1. Sediments from the grab samples are emptied into a tub and then passed through sieves. The appropriate sieve sizes should be selected based on sediment type and organisms to be collected.

**ERL-N STANDARD OPERATING PROCEDURE
BENTHIC ORGANISM COLLECTION**

ERL-N SOP 1.02.001
REVISION 0
FEBRUARY 1990
PAGE 3 OF 4

2. The worms are picked from the sieves with forceps and rinsed in Carolina bowls (or other container) with clean seawater.
3. Any attached sediment should be removed from the organism prior to adding it to the sample jar. Gently spraying with a the hose can generally remove sediments remaining after rinsing.
4. Worms should be segregated by species if more than one species is being collected.
5. Typically, a minimum of 10g of biomass for each species is required for chemical analysis.

B. Quahogs

1. Hire a professional quahog fisherman. Clifford Adams of North Kingstown, RI and Jack Gaines Jr. of Jamestown, RI have cooperated previously and have indicated a desire to work again in the future. Art Ganz of Rhode Island DEM has assisted in the past with locating fisherman willing to cooperate. It is best if only one person accompanies the fisherman as space on board a typical quahog boat is limited. It is a good practice to pay the fisherman at the completion of the day rather than make him/her wait for the laboratory's petty cash system.
2. A chart with the station locations is essential in order to minimize any potential for miscommunication.
3. Sample bags should be prelabelled and organized so as not to waste time in the field.

C. *Mytilus*

In 1989 dense populations of mussels were found on the flanks of the channel to the west and southwest of Prudence Island. Mussels can also be found attached to rocks and other objects in intertidal areas and can be collected by prying or raking.

1. Deploy the scallop dredge (~5:1 ratio of line to depth) and tow at approximately 2-3 knots for 5-10 minutes.
2. Haul the dredge back, dump the catch on board, redeploy the dredge, and sort the catch.

D. Oysters

Collections should be conducted at low tide. Some locations may be accessible by foot, ie. Allen Harbor landfill; others will require a boat, ie. Prudence Island. Oysters can be found attached to rocks or other hard substrates/objects, in brackish low intertidal and subtidal areas. Some wading may be necessary when collecting oysters.

1. Pry oysters from the object, with a pocket knife or other prying implement.

E. Soft-shell clams

Collections must be conducted at low tide, preferably during a spring tide (new or full moon) when the tidal range is greatest thus allowing more time for collecting. Soft-shells are generally found closer to the surface in the summer than in the winter. As with oysters, some sites will be accessible by foot whereas others will require a shallow draft boat (whaler-yes, West Passage-no).

1. Locate siphon holes by walking along the intertidal region of the flat (primarily the lower 1/3 of the intertidal area).
2. Once siphon holes are located commence digging. Care should be taken to avoid breaking the "soft-shell" clams during excavation. One trick to avoid breaking the clams is to dig a hole several inches away from the siphon holes, insert the clam fork beyond the siphon hole and "pull" the sand/mud into the previous hole, then pick through the sand/mud for the clam(s).

IV. TROUBLE SHOOTING

Discussed in methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
CAGED BIVALVE DEPLOYMENT**

**ERL-N SOP 1.02.002
REVISION 0
MARCH 1990
PAGE 1 OF 4**

POINT OF CONTACT:

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I. OBJECTIVE

Deployment and retrieval of caged bivalves for environmental monitoring.

II. NECESSARY MATERIALS AND EQUIPMENT

- 17' Whaler or larger vessel
 - Coolers for holding the organisms during transport
 - Twine
 - Pocket knife
 - Foul weather gear
 - First Aid Kit
 - Mussel trawls (per trawl, see schematic):
 - 4 cinder blocks
 - 4 trawl floats
 - 4 mussel baskets
 - 2 lobster trap buoys
 - 2 small (1lb) weights
 - 1 trawl line, 1/2 - 3/4 inch polypropylene, approximate length = 2 x maximum depth + 20m.
- NOTE--There are no hard and fast rules regarding the length of the trawl line, flexibility permits adaptation to the various constraints encountered at any particular deployment location. One should make certain that under no circumstances will excess line be found floating at or near the surface.**
- 4 leaders, 2-3m lengths of the same line as the trawl line
 - 4 plastic ties
 - 4 2-3m lengths of "tire cord" (small diameter nylon line)

III. METHODS

**ERL-N STANDARD OPERATING PROCEDURE
CAGED BIVALVE DEPLOYMENT**

ERL-N SOP 1.02.002
REVISION 0
MARCH 1990
PAGE 2 OF 4

In order to facilitate transport and deployment, it is recommended that trawl lines be partially assembled before going into the field. Prepare the trawl line but do not attach cinder blocks, trawl floats, or mussel baskets until just prior to deployment.

A. Trawl line construction

At the laboratory/on land:

1. After consulting nautical charts, cut the desired length of line (minimum of 2 x the high water depth + 20m).
2. Attach one buoy to each end, one or two overhand knots above and below the buoy should be sufficient. A loop of some sort above the buoy will facilitate retrieval with a boat hook.
3. Attach the two small weights to the main trawl line 2-3m below the two buoys--this prevents line from floating at the surface during low tide.
4. Attach (by knotting or splicing) the 4 leaders to the main trawl line. Attach the first at the maximum total depth + 2m, and the rest at consecutive 5m intervals.
5. Coil trawl for transport to the field. Securing the coil with twine can make a bulky trawl more manageable.
6. Attach one 2-3m section of "small" nylon line (tire cord) to each of the trawl floats. Make certain that the length of line is appropriate for the trawl floats be suspended 1m above the substrate. Tie them together in sets of four for transport.

In the field:

7. Stand 4 cinder blocks upright in a line position near the point from which they will be deployed.
8. Uncoil the trawl line and attach one cinder block to the end of each of the leaders using a clove hitch. The clove hitch can be secured with either a half hitch or by twisting open the braid of the line above the clove hitch and passing the free end of the line through the braid.
9. Secure the trawl floats to the trawl line just above the knot at each cement block, again use a clove hitch. The trawl float should float 1m above the substrate when deployed.
10. Using the plastic ties, attach the mussel basket to the trawl float.

B. Deployment

1. Deploy the trawl sequentially beginning with one buoy then each of the cinder blocks and finally the second buoy. Be deliberate. Lower each block into the water, making

**ERL-N STANDARD OPERATING PROCEDURE
CAGED BIVALVE DEPLOYMENT**

**ERL-N SOP 1.02.002
REVISION 0
MARCH 1990
PAGE 3 OF 4**

certain that the trawl float and the mussel basket float free, and slowly pay out the line by hand until reaching the next block. Repeat the procedure for each block. Avoid dropping the blocks on top of one another by maneuvering the boat in low speed or by using the prevailing wind to stretch the trawl as it is deployed. Once deployed, it may be necessary to tow one end of the trawl in order to fully stretch the trawl.

C. Retrieval

1. This is essentially deployment in reverse. Pick up the "down wind" buoy.
2. Pull the trawl up one block at a time.
3. Once the entire trawl is on deck, disassemble placing the mussel baskets in coolers, piling the cinder blocks together, tying the trawl floats together, and coiling the trawl.
4. Retrieve the next trawl.

IV. TROUBLE SHOOTING/HINTS

1. Set trawls 90deg to the predominant currents when ever possible as this helps to minimize tangling.
2. Avoid setting the trawl in a channel, mooring area or other high use area.
3. Avoid deploying mussel trawls near lobster trawls--they may not be there when you return.
4. Notify the local Harbormaster and appropriate regulatory agencies prior to deployment.
5. It may be necessary to make adjustments to the trawls in the field; therefore, it is worthwhile to bring extra line, blocks, and buoys if space permits. For example, where there is not enough space to deploy an entire trawl, it may be feasible to deploy each block individually, thus requiring extra line and floats.
6. In high energy zones with hard bottoms it may be necessary to use heavier/denser weights than cinder blocks (attaching lengths of chain to the trawl line between the cinder blocks may be sufficient). Whenever possible, use the wind, don't fight it.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

**ERL-N STANDARD OPERATING PROCEDURE
CAGED BIVALVE DEPLOYMENT**

**ERL-N SOP 1.02.002
REVISION 0
MARCH 1990
PAGE 4 OF 4**

None.

**ERL-N STANDARD OPERATING PROCEDURE
MYSID CAGE CONSTRUCTION AND FIELD DEPLOYMENT**

ERL-N SOP 1.02.003
REVISION 0
MAY 1990
PAGE 1 OF 8

POINT OF CONTACT:

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I. OBJECTIVE

Deployment and retrieval of caged mysids for environmental monitoring.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Cage Construction

- Sheet plexiglass (0.25 in thick)--for construction of mysid cage:
 - Fin (see template, Fig. 3)
 - Frame (all 2 cm wide):
 - 1 - 48 cm
 - 1 - 36 cm
 - 2 - 41 cm
 - 1 - 29 cm
 - 1 - 22 cm
 - 3 - 14 cm
- Polycarbonate core liner [3 in (2.75) outside diameter (O.D.)]:
 - 10 - 4 cm long (chambers)
 - 20 - 2 cm long (covers)
 - 20 - 0.5 cm long (protective rims)
- Saw and hand drill - to cut, drill, and sand plexiglass
- Methylene chloride - to attach pieces of plexiglass to each other
- Core liner caps - 20
- Nitex plankton netting (500 μ m) - 400 in²
- Bronze quick-snap swivel (3.25 in.)
- High-impact trawl floats (8 in.)
- Hot-dip galvanized chain (0.25 in.) - 8 ft.
- Cinder block "anchor"
- Polypropylene 3-strand rope (0.5 in.)
- Braided Dacron rope (0.25 in.) - 12 ft.

**ERL-N STANDARD OPERATING PROCEDURE
MYSID CAGE CONSTRUCTION AND FIELD DEPLOYMENT**

ERL-N SOP 1.02.003
REVISION 0
MAY 1990
PAGE 2 OF 8

B. Mysid deployment

- Juvenile (7-day old) mysids - 50 per station
- Clean seawater
- Foul weather gear
- Boots
- Gloves (for protection against contaminated water)
- Water proof notebook
- Water proof markers
- Standard safety equipment
- First Aid Kit
- Boat of appropriate size for deployment of mysids
- Compass

C. Monitoring Cages

- Toothbrush - for cleaning netting

D. Retrieving cages

- No additional equipment

E. Endpoint determination

- Light table
- Wide-bore glass pipette and bulb
- Forceps
- Depression slide
- Dissecting microscope
- Source of deionized water
- Pre-weighed small (5-10 mg) aluminum pans
- Drying oven (60° C)
- Laboratory balance capable of weighing to 0.001 mg.
- Desiccator and desiccant

III. METHODS

A. Cage construction (Fig. 1)

1. Cut plexiglass sheet and core liner pipe to lengths listed above (scrape or sand all sharp edges).
2. Cut out a 16 mm section from each cover and rim. Sand cut edges until covers fit snugly inside chambers. "Glue" (methylene chloride) cover edges together to form a complete ring. Glue 16 mm pieces onto the center of the inside wall of each chamber to act as stops for covers. Sand cut edges of rims until the O.D. of each

**ERL-N STANDARD OPERATING PROCEDURE
MYSID CAGE CONSTRUCTION AND FIELD DEPLOYMENT**

ERL-N SOP 1.02.003
REVISION 0
MAY 1990
PAGE 3 OF 8

matches the O.D. of the covers. Glue rim edges together to form a complete ring.

3. Cut Nitex netting into 20 4 x 4 in squares. Glue netting to one side of each cover by sandwiching between cover and rim, applying methylene chloride and clamping.
4. Arrange chambers as shown in drawing. Cut and sand plexiglass strips for frame until each chamber contacts the frame (in 3 places for outside chambers, 2 places for inside chambers). Leave a ¼" space between adjacent chambers to facilitate removal of covers. Bevel edges where frame members meet.
5. Elevate frame members so that they contact the mid-point of each chamber. Glue all points where frame members meet and chambers contact frame.
6. Glue 48 cm reinforcement strip to frame. Bend (over frame) and glue 14 cm reinforcement strips (2) to frame. Triangular plexiglass corner reinforcements may be used also.
7. Drill angled holes through frame flanges. Bevel and smooth hole edges.
8. Glue fin to top of cage with square plexiglass reinforcements.
9. Tie one end of 1 ft length of braided ¼" rope to bottom of cage and other end to swivel.
10. Tie both ends of two 5 ft lengths of braided ¼" rope to the trawl float and opposite ends to each corner frame flange.

B. Cage deployment (Fig. 2)

1. Place cover and core liner cap on upcurrent side of each chamber. Fill each chamber with clean seawater.
2. Place 5 test organisms in each chamber.
3. Place cover on downcurrent side of each chamber. Fill each chamber to the top of rim with clean seawater. Puncture a small hole in the center of each downcurrent core liner cap to allow excess water and air to escape. Place these caps on the downcurrent side of each chamber.
4. Transport caged mysids to deployment site. Maintain or acclimate to ambient seawater temperatures.
5. Tie middle of 9 ft length of braided rope to trawl float. Feed one end through each angled hole in the frame flanges and tie off.
6. Weave one end of polypropylene rope through 8 ft of ¼" chain. Tie this end to

cinder block anchor. Tie a loop in the other end of the polypropylene rope and clip the swivel onto the loop.

7. Lower the block, rope and cage into the water.
8. Reach beneath the water's surface and remove caps starting with the bottom chamber and working towards the chambers at the top of the cage.
9. After all caps are removed, shake cage vigorously to remove any trapped air.

C. Monitoring cages

1. Brush cage netting with toothbrush every 24-48 hrs. (depending on amount of fouling) to remove debris.

D. Retrieving cages

1. Cap both sides of each chamber below the water's surface (caps with holes on the downstream side).
2. Transport caged mysids to laboratory. Maintain ambient seawater temperatures.

E. Endpoint determination

1. Place cage upcurrent side down on light table. Remove core liner caps and covers from downcurrent side of each chamber.
2. % Survival - remove and count mysids from each chamber with wide-bore glass pipette and bulb.
3. % Females with eggs - examine each mysid individually in a depression slide (under a dissecting scope at 240x magnification); determine sex and presence or absence of eggs for females.
4. Mean individual weight - place DI-rinsed mysids from each replicate chamber on tared aluminum weighing pans. Place pans in drying oven (60° C) for 48 hours and weigh again.

IV. TROUBLE SHOOTING

1. Avoid deploying mysid cages in a channel, mooring area, or other high use area.

**ERL-N STANDARD OPERATING PROCEDURE
MYSID CAGE CONSTRUCTION AND FIELD DEPLOYMENT**

ERL-N SOP 1.02.003
REVISION 0
MAY 1990
PAGE 5 OF 8

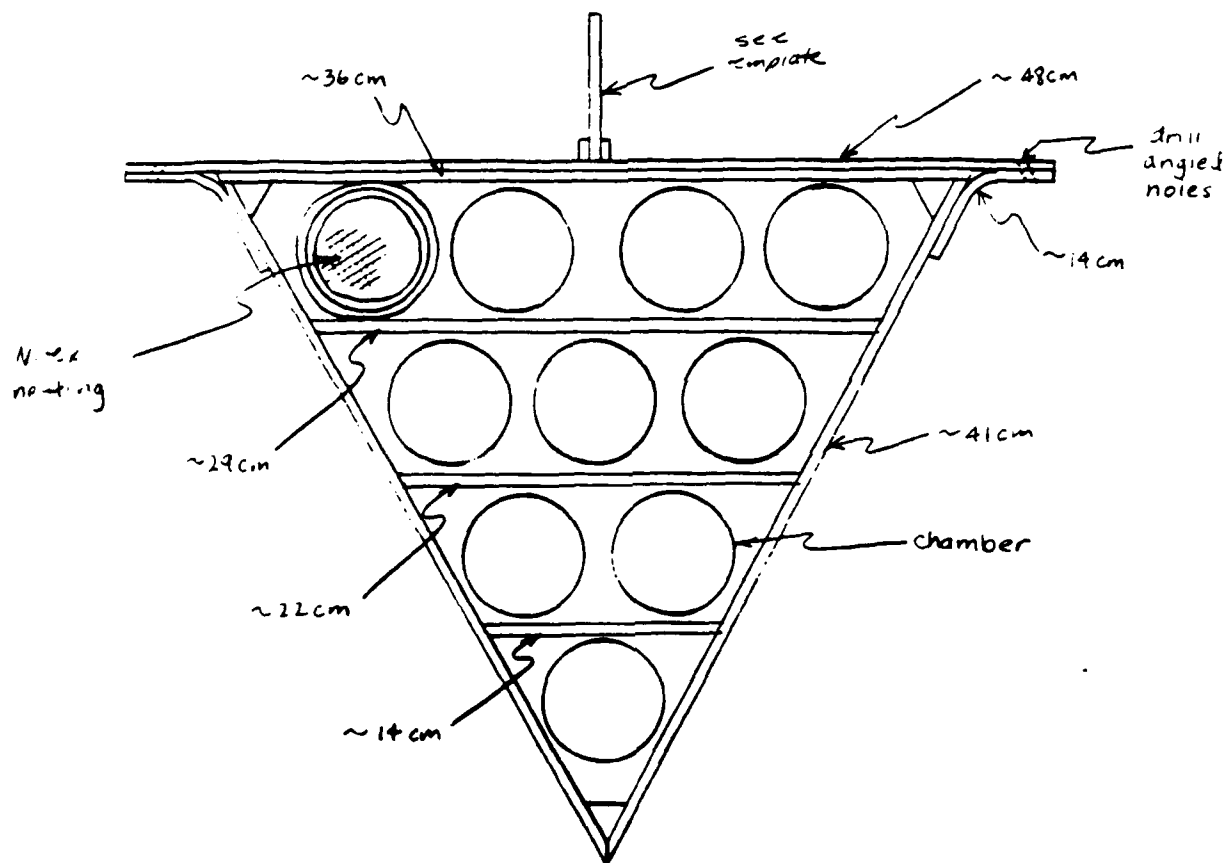
2. Avoid deploying mysid cages near lobster trawls.

V. STATISTICAL ANALYSIS AND DATA USAGE

Data are analyzed by pairwise comparison of endpoints from each station to a control by one-way analysis of variance (ANOVA).

VI. REFERENCES

US EPA. 1988. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. US EPA Office of Research and Development, EPA/600/4-87/028.



TOP

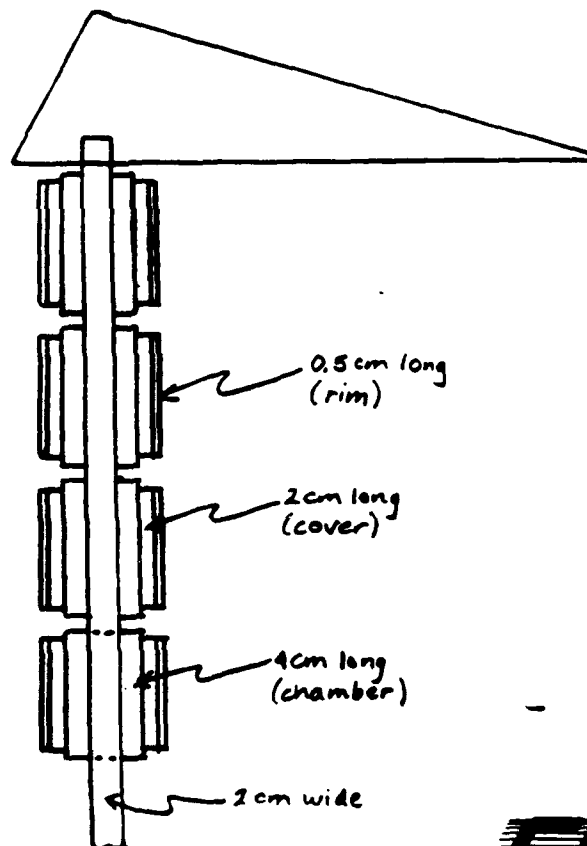


Fig. 1

BOTTOM

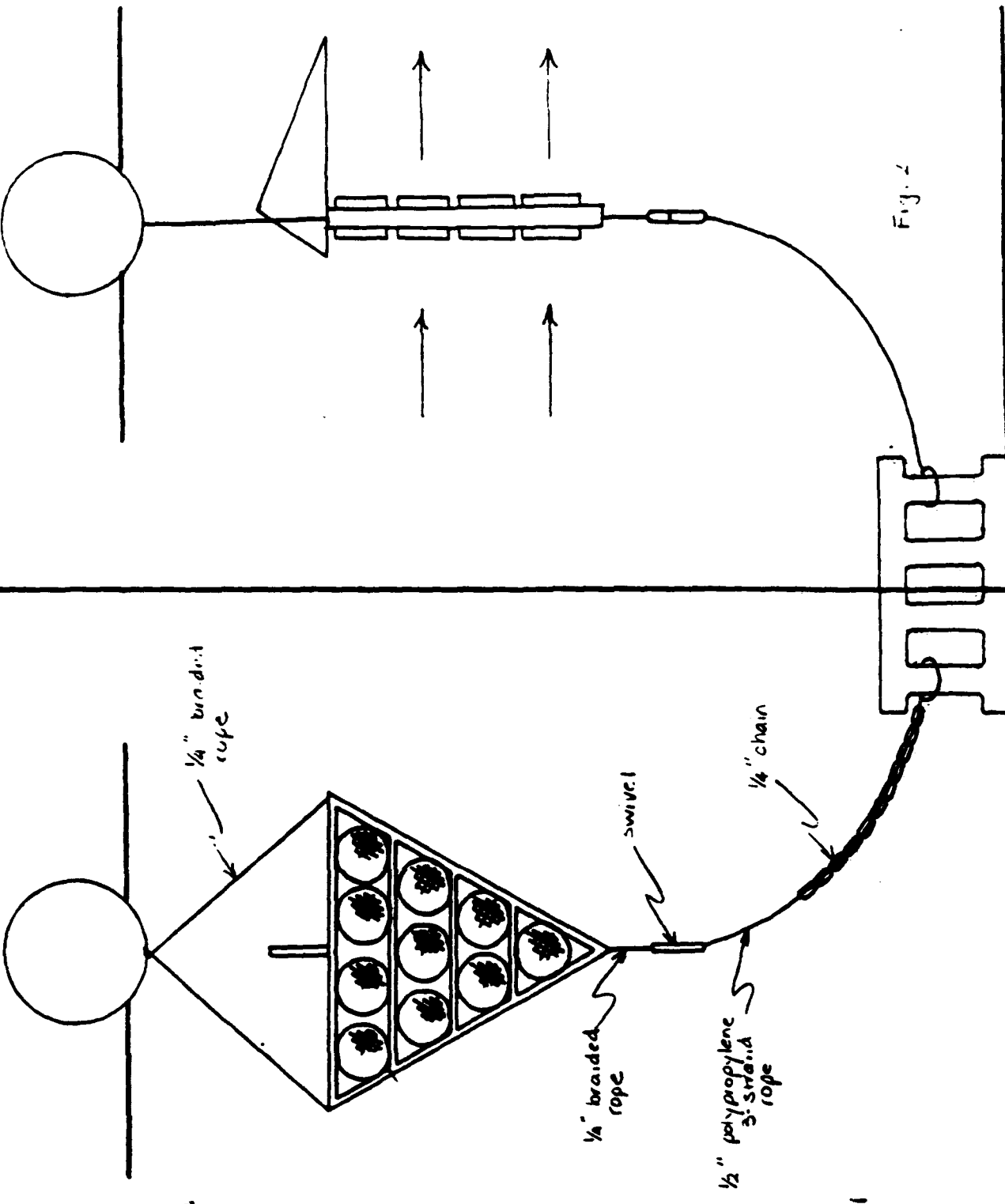


Fig. 2

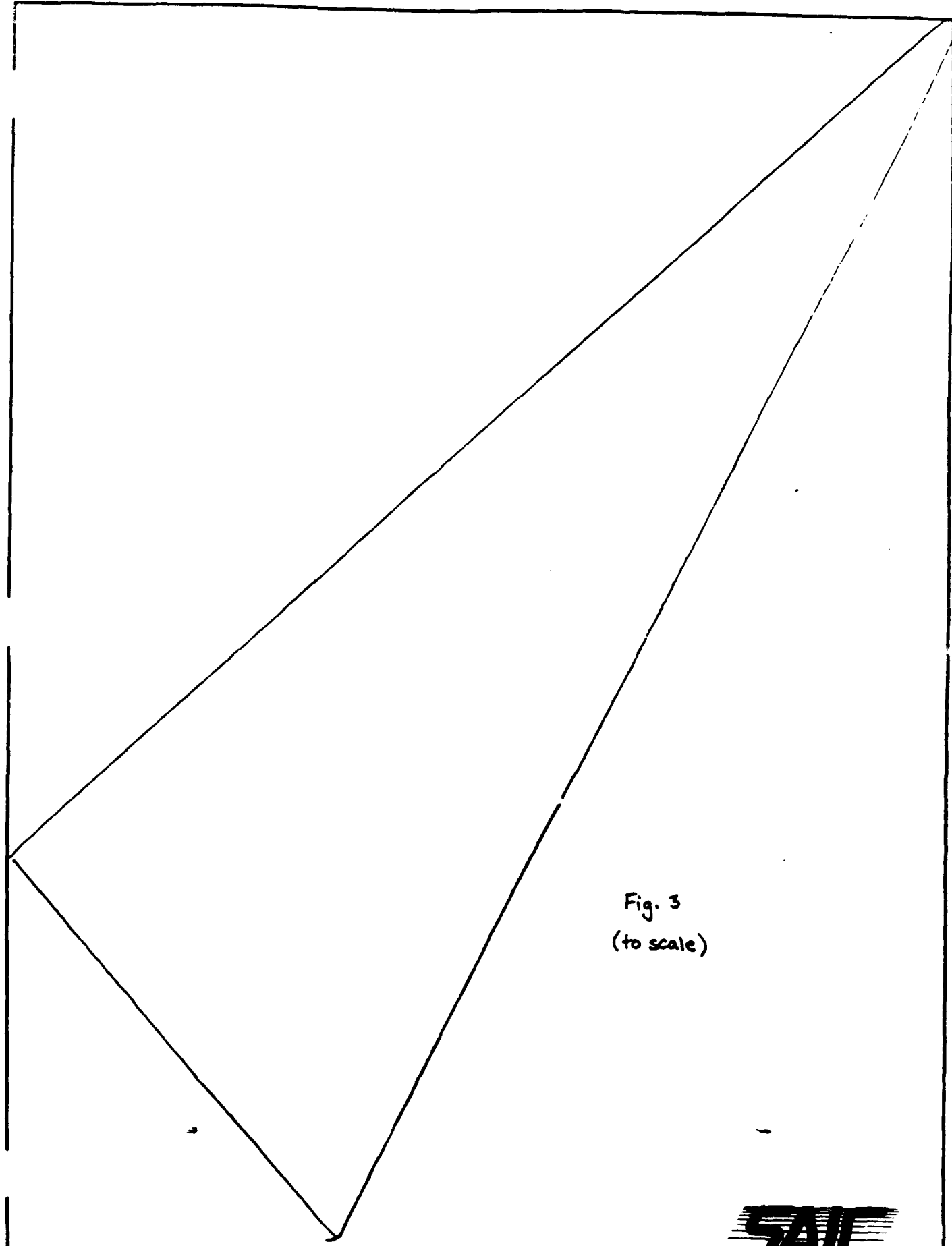


Fig. 3
(to scale)

**ERL-N STANDARD OPERATING PROCEDURE
SUSPENDED SOLIDS DETERMINATION IN
WATER SAMPLES**

**ERL-N SOP 1.02.004
REVISION 0
DECEMBER 1990
PAGE 1 OF 3**

POINT OF CONTACT:

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I. OBJECTIVE

To determine the suspended solids concentration of water column samples. The practical range of the determination is 4 mg/l to 20,000 mg/l.

II. NECESSARY MATERIALS AND EQUIPMENT

- Plastic sampling container, 1 L
- Pre-weighed, pre-washed 0.45 um glass fiber filters without organic binder, such as Millipore AP-40, Reeves Angel 934-AH, Gelman type A/E, or equivalent. NOTE: Because of the physical nature of glass fiber filters, the absolute pore size cannot be controlled or measured. Terms such as "pore size", collection efficiencies and effective retention are used to define this property in glass fiber filters. Values for these parameters vary for the filters listed above.
- Forceps
- Small containers or dishes to hold individual filters
- Filtration apparatus with reservoir and a coarse (40-60 microns) fritted disc as a filter support. NOTE: Many funnel designs are available in glass or porcelain. Some of the most common are Hirsch or Buchner funnels, membrane filter holders and Gooch crucibles. All are available with coarse fritted disc.
- Desiccator with desiccant
- Laboratory notebook
- Suction flask
- Drying oven, 103-105 °C
- Analytical balance, capable of weighing to 0.1 mg

III. METHODS

A. Preparation of Glass Fiber Filter Disc

1. Place the glass fiber filter on the membrane filter apparatus or insert into bottom of a suitable Gooch crucible with wrinkled surface up.
2. While vacuum is applied, wash the disc with three successive 20 ml volumes of distilled water.

**ERL-N STANDARD OPERATING PROCEDURE
SUSPENDED SOLIDS DETERMINATION IN
WATER SAMPLES**

ERL-N SOP 1.02.004
REVISION 0
DECEMBER 1990
PAGE 2 OF 3

3. Remove all traces of water by continuing to apply vacuum after water has passed through.
4. Remove filter from membrane filter apparatus or both crucible and filter if Gooch crucible is used, and dry in an oven at 103-105 °C for one hour.
5. Remove to desiccator and store until needed.
6. Repeat the drying cycle until a constant weight is obtained (weight loss is less than 0.5 mg).
7. Weigh immediately before use. After weighing, handle the filter or crucible/filter with forceps or tongs only.

B. Selection of Sample Volume

1. For a 4.7 cm diameter filter, filter 100 ml of sample.
 - a. If weight of captured residue is less than 1.0 mg, the sample volume must be increased to provide at least 1.0 mg of residue.
 - b. If other filter diameters are used, start with a sample volume equal to 7 ml/cm of filter area and collect at least a weight of residue proportional to the 1.0 mg stated above.
2. If during filtration of this initial volume the filtration rate drops rapidly, or if filtration time exceeds 5 to 10 minutes, the following scheme is recommended:
 - a. Use an unweighed glass fiber filter of choice affixed in the filter assembly.
 - b. Add a known volume of sample to the filter funnel and record the time elapsed after selected volumes have passed through the filter. Twenty-five ml increments for timing are suggested.
 - c. Continue to record the time and volume increments until filtration rate drops rapidly. Add additional sample if the filter funnel volume is inadequate to reach a reduced rate.
 - d. Plot the observed time versus volume filtered.
 - e. Select the proper filtration volume as that just short of the time at which significant change in filtration rate occurred.

C. Filtering a Sample

1. Assemble the filtering apparatus and begin suction.
2. Wet the filter with a small volume of distilled water to seat it against the fritted

**ERL-N STANDARD OPERATING PROCEDURE
SUSPENDED SOLIDS DETERMINATION IN
WATER SAMPLES**

ERL-N SOP 1.02.004
REVISION 0
DECEMBER 1990
PAGE 3 OF 3

support.

3. Shake the sample vigorously and quantitatively transfer the predetermined sample volume selected in Section B to the filter using a graduated cylinder. Remove all traces of water by continuing to apply vacuum after sample has passed through.

4. With suction on, wash the graduated cylinder, filter, non-filterable residue and filter funnel wall with three portions of distilled water allowing complete drainage between washing. Remove all traces of water by continuing to apply vacuum after water has passed through.

NOTE: Total volume of wash water used should equal approximately 2 ml per cm. For a 4.7 cm filter the total volume is 30 ml.

5. Carefully remove the filter from the filter support. Alternatively, remove crucible and filter from crucible adapter.

6. Dry at least one hour at 103-105 °C.

7. Cool in a desiccator and weigh.

8. Repeat the drying cycle until a constant weigh is obtained (weight loss is less than 0.5 mg).

IV. TROUBLE SHOOTING

1. Avoid prolonged exposure of dried filters to air, as the filters will tend to collect moisture from the air.

2. Preservation of the sample is not practical; analysis should begin as soon as possible. Refrigeration or icing to 4 °C, to minimize microbiological decomposition of solids, is recommended.

V. STATISTICAL ANALYSIS AND DATA USAGE

Calculate non-filterable residue as follows:

$$\text{Non-filterable residue, mg/l} = \frac{(A - B) \times 1,000}{C}$$

where: A = weight of filter (or filter and crucible) + residue in mg

B = weight of filter (or filter and crucible) in mg

C = ml of sample filtered

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
WATER COLUMN SALINITY, CONDUCTIVITY,
TEMPERATURE, AND DISSOLVED OXYGEN
DETERMINATION**

**ERL-N SOP 1.02.005
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PAGE 1 OF 2**

POINT OF CONTACT:

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I. OBJECTIVE

Quantification of salinity, conductivity, temperature, and dissolved oxygen levels in the water column.

II. MATERIALS AND EQUIPMENT

- Refractometer
- SBE25 Sealogger CTD and associated necessary equipment (see operating manual)
- Hydrolab DataSond[®]3 and associated necessary equipment (see operating manual)
- Thermometer
- Nester 8500 portable dissolved oxygen meter (D.B. 577032) and BOD electrode (Nester no. 617032) (see manuals for meter and electrode)

III. METHODS

The methods used by ERL-N to determine water column salinity, conductivity, temperature, and dissolved oxygen are widely used. Most of these methods are related to the use of a specific instrument and are provided to the purchaser of the instrument by the manufacturer. These methods or instructions must be followed carefully in order to properly quantify the water column parameters listed above.

IV. TROUBLE SHOOTING

Not applicable.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

Hydrolab DataSond[®]3 Operating Manual (and Performance Manual). April 1990, Hydrolab

**ERL-N STANDARD OPERATING PROCEDURE
WATER COLUMN SALINITY, CONDUCTIVITY,
TEMPERATURE, AND DISSOLVED OXYGEN
DETERMINATION**

**ERL-N SOP 1.02.005
REVISION 0
FEBRUARY 1991
PAGE 2 OF 2**

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Nester Instruments (A Div. of Leeds and Northrup). PO Box 1187, Millville,
NJ 08332. (609)825-0254.

SBE25 Sealogger CTD Operating Manual. April 1990. Sea-Bird Electronics, Inc. 1808 136th
Place NE Bellevue, Washington 98005. (206)643-9866.

ERL-N STANDARD OPERATING PROCEDURE
Champia parvula SEXUAL REPRODUCTION TEST

ERL-N SOP 1.03.001
REVISION 0
JANUARY 1991
PAGE 1 OF 13

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I. OBJECTIVE

This method measures the effects of toxic substances in effluents and receiving water on the sexual reproduction of the marine macroalga, *Champia parvula*, during a two day exposure.

II. NECESSARY MATERIALS AND EQUIPMENT

- Facilities for holding and acclimating test organisms.
- Laboratory *Champia parvula* culture unit -- See culturing methods below. To test effluent or receiving water toxicity, sufficient numbers of sexually mature male and female plants must be available.
- Environmental chamber or equivalent facility with temperature ($23 \pm 1^{\circ}\text{C}$) and light ($100 \text{ uE/m}^2/\text{s}$, or 500 ft-c) control.
- Water purification system -- Millipore Super-Q, deionized water (DI) or equivalent.
- Air pump -- for supplying uncontaminated air.
- Air lines, and air stones -- for aerating cultures.
- Balance -- Analytical, capable of accurately weighing to 0.0001 g.
- Reference weights, Class S -- for checking performance of balance.
- pH meter -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of this parameter, a portable, field-grade instrument is acceptable.
- Dissecting (stereoscope) microscope -- for counting cystocarps.
- Compound microscope -- for examining the condition of plants.
- Count register, 2-place -- for recording cystocarp counts.
- Rotary shaker -- for incubating exposure chambers (hand-swirling twice a day can be substituted).
- Drying oven -- to dry glassware.
- Filtering apparatus -- for use with membrane filters (47mm).
- Refractometer -- for determining salinity.
- Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

- Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.
- Beakers -- Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.
- Erlenmeyer flasks, 250 mL, or 200 mL disposable polystyrene cups, with covers -- for use as exposure chambers.
- Bottles -- borosilicate glass or disposable polystyrene cups (200-400 mL) for use as recovery vessels.
- Wash bottles -- for deionized water, for rinsing small glassware and instrument electrodes and probes.
- Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.
- Micropipettors, digital, 200 and 1000 μ L -- to make dilutions.
- Pipets, volumetric -- Class A, 1-100 mL.
- Pipetter, automatic -- adjustable, 1-100 mL.
- Pipets, serological -- 1-10 mL, graduated.
- Pipet bulbs and fillers -- PROPIPET[®], or equivalent.
- Forceps, fine-point, stainless steel -- for cutting and handling branch tips.
- Mature *Champia parvula* plants.
- Sample containers -- for sample shipment and storage.
- Petri dishes, polystyrene -- to hold plants for cystocarp counts and to cut branch tips. Other suitable containers may be used.
- Disposable tips for micropipettors.
- Aluminum foil, foam stoppers, or other closures -- to cover culture and test flasks.
- Tape, colored -- for labelling test chambers.
- Markers, water-proof -- for marking containers, etc.
- Data sheets (one set per test) -- for data recording.
- Buffers, pH 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check.
- Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms).
- Effluent and/or surface water.
- Saline test and dilution water -- The use of natural seawater is recommended for this test. A recipe for the nutrients that must be added to the natural sea water is given in Table 1.
- Artificial seawater -- Artificial seawater (GP-2) preparation is described in Table 2.

III. METHODS

A. *Champia parvula* Cultures

1. The adult plant body (thallus) of *C. parvula* is hollow, septate, and highly branched. New cultures can be propagated asexually from excised branches, making it possible to maintain clonal material indefinitely.
2. Stock cultures of both male and female plants are maintained in separate, aerated, 1-L Erlenmeyer flasks, or equivalent, containing 800 mL of culture medium. Several

cultures of both males and females are maintained simultaneously to keep a constant supply of plant material available. New cultures should be started weekly, so that plants are available in different stages of development (i.e., with different amounts of tissue per flasks). The total number of cultures maintained will depend on the expected frequency of testing.

3. Prior to use in toxicity tests, stock cultures should be examined to determine their condition. Females can be checked by examining a few branch tips under a compound microscope (100 X or greater). Several trichogynes (reproductive hairs to which the spermatia attach) should be easily seen near the apex (Figure 1).

4. Male plants should be visibly producing spermatia. This can be checked by placing some male tissue in a petri dish, holding it against a dark background and looking for the presence of spermatial sori. Mature sori can also be easily identified by looking along the edge of the thallus under a compound microscope (Figures 2 and 3).

5. A final, quick way to determine the relative "health" of the male stock culture is to place a portion of a female plant into some of the water from the male culture for a few seconds. Under a compound microscope numerous spermatia should be seen attached to both the sterile hairs and the trichogynes (Figure 4).

6. Culture medium prepared from natural seawater is preferred (Table 1). However, as much as 50% of the natural seawater may be replaced by the artificial seawater (GP-2) described in Table 2.

7. The seawater is autoclaved for 20 min at 15 psi. The culture flasks are capped with aluminum foil and autoclaved dry, for 10 min. Culture medium is made up by dispensing seawater into sterile flasks and adding the appropriate nutrients from a sterile stock solution.

8. Alternately, 1-L flasks containing seawater can be autoclaved. Sterilization is used to prevent microalgal contamination, and not to keep cultures bacteria free.

B. Test Procedures

1. Test Solutions

a. The salinity of the test water must be 30 ‰, and vary no more than ± 2 ‰ among replicates. In addition, effluent test solutions must be at least 50% natural seawater. Therefore, the maximum effluent concentration which can be tested is 50% (if the salinity of the effluent is adjusted by adding the dry salt formulation described in Table 2). The salinity of the effluent may also be adjusted by adding brine prepared from natural seawater (100 ‰, see SOP on Brine) or concentrated (triple strength) salt solution (GP-2 described in Table 2). Control solutions should be prepared with the same percentage of natural seawater and at the same salinity (using deionized water adjusted with dry salts, or brine) as used for the effluent dilutions.

b. The selection of the effluent test concentrations should be based on the objectives of the test. Two commonly used dilution factors are 0.5 and 0.3. If the effluent is known or suspected to be highly toxic, a range of effluent concentrations with a low (ie- 10%) maximum effluent concentration should be used.

c. The volume of effluent required for the test using a 0.3 dilution series is approximately 1200 mL. The test should begin as soon as possible after sample collection, preferably within 24 hr. Sample holding time should not exceed 36 hours.

d. Prepare enough test solution at each effluent concentration to provide 100 mL of solution for each of three replicate test chambers.

e. The formula for the enrichment for natural seawater dilution water is listed in Table 1. Both EDTA and trace metals have been omitted. This formula should be used for the 2-day exposure period, but it is not critical for the recovery period.

2. Preparation of Plants for the Test

a. Plant cuttings from healthy cultures should be prepared for the test using fine-point forceps, with the plants in a small amount of seawater in a petri dish. Prepare the female cuttings first to minimize the chances of contaminating them with water containing spermata from the male stock cultures.

b. For female plants, five cuttings, severed 7-to-10 mm from the ends of the branch, should be prepared for each treatment chamber. Try to be consistent in the number of branch tips on each cutting.

c. For male plants, one cutting, severed 1.5-to-2 cm from the end of the branch, is prepared for each treatment chamber.

3. Performing the Test

a. The temperature of the effluent sample should be adjusted to that of the test ($23 \pm 1^{\circ}\text{C}$) and maintained at that temperature until portions are added to the dilution water.

b. Label three test chambers per effluent treatment and for the controls.

c. Fill the test chambers with 100 mL of control or treatment water (28 - 32 ‰). For reference toxicant tests, all test chambers can be filled with control water and the toxicant added with a pipette. For toxicant volumes exceeding 1 mL, adjust the amount of dilution water to give a final volume of 100 mL.

d. Add five female branches and one male branch to each test chamber. The toxicant must be present before the male plant is added.

- e. Place the test chambers under cool-white light (approx. 100 $\mu\text{E}/\text{m}^2/\text{s}$, or 500 ft-c), with a photoperiod of 16 h light and 8 h darkness. Maintain the temperature between 22 and 24°C. Check the temperature by placing a laboratory or recording thermometer in a flask of water among the test chambers. Record the temperature daily.
- f. Gently hand-swirl the chambers twice a day, or shake continuously at 100 rpm on a rotary shaker.
- g. Label the recovery vessels. These vessels can be almost any type of container or flask containing 100 - 200 mL of seawater and nutrients (see Tables 1 and 2).
- h. Using forceps, gently remove the female branches from the test chamber and place into the recovery bottles. Add aeration tubes and foam stoppers.
- i. Place the vessels under cool-white light (at the same irradiance as the stock cultures) and aerate for the 5- to 7-day recovery period. If a shaker is used, do not aerate the solutions (this will enhance the water motion).
- j. At the end of the recovery period, count the number of cystocarps (Figures 5, 6, and 7) per female and record the data (Figure 8).
 1. Place the female plants between the inverted halves of a polystyrene petri dish or other suitable containers with a small amount of seawater (to hold the entire plant in one focal plane).
 2. Count the cystocarps under a stereomicroscope, and are distinguished from young branches because they possess an apical opening for spore release (ostiole) and darkly pigmented spores.
- k. A test is not acceptable if:
 1. Control mortality exceeds 20% (generally there is no control mortality).
 2. Control plants average less than 10 cystocarps.

IV. TROUBLE SHOOTING

1. Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment.
2. Adverse effects of high concentrations of suspended and/or dissolved solids, and extremes of pH, may mask the presence of toxic substances.

3. Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

4. If there is uncertainty about the identification of an immature cystocarp, aerate the plants for another 1 or 2 days. After this additional aeration, the presumed cystocarp will either look more like a mature cystocarp or a young branch, or will have changed very little if at all (i.e., an aborted cystocarp). No new cystocarps will form since the males have been removed, and the plants will only get larger. Occasionally, cystocarps will abort, and these should not be included in the counts. Aborted cystocarps are easily identified by their dark pigmentation and often by the formation of a new branch at the apex.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data.
2. Statistically significant differences in the mean number of cystocarps are determined by one-way analysis of variance (ANOVA) followed by Dunnett's Procedure (Dunnett, 1955) to generate no effect and least effect concentrations (NOECs and LOECs).

VI. REFERENCES

- Dunnett, C.W. 1955. A multiple comparisons procedure for comparing several treatments with a control. JASA 50:1096-1101.
- US EPA. 1988. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. Weber, C.I., et al (eds). EPA Office of Research and Development EPA-600/4-87/028 (May 1988).

TABLE 1. NUTRIENTS TO BE ADDED TO NATURAL SEAWATER AND TO ARTIFICIAL SEAWATER (GP-2) DESCRIBED IN TABLE 2. THE CONCENTRATED NUTRIENT STOCK SOLUTION IS AUTOCLAVED FOR 15 MIN (VITAMINS ARE AUTOCLAVED SEPARATELY FOR 2 MIN AND ADDED AFTER THE NUTRIENT STOCK SOLUTION IS AUTOCLAVED). THE PH OF THE SOLUTION IS ADJUSTED TO APPROXIMATELY PH 2 BEFORE AUTOCLAVING TO MINIMIZE THE POSSIBILITY OF PRECIPITATION.

	Amount of Reagent Per Liter of Concentrated Nutrient Stock Solution	
	Stock Solution For Culture Medium	Stock Solution For Test Medium
<u>Nutrient Stock Solution¹</u>		
Sodium Nitrate (NaNO ₃)	6.35 g	1.58 g
Sodium Phosphate (NaH ₂ PO ₄ · H ₂ O)	0.64 g	0.16 g
Na ₂ EDTA · 2 H ₂ O	133 mg	- -
Sodium Citrate (Na ₃ C ₆ H ₅ O ₇)	51 mg	12.8 mg
<u>Iron²</u>		
<u>Vitamins³</u>		

¹Add 10 mL of appropriate nutrient stock solution per liter of culture or test medium.

²A stock solution of iron is made that contains 1 mg iron/mL. Ferrous or ferric chloride can be used. Add 9.75 mL of the iron stock solution to each liter of culture medium and 2.4 mL to each liter of test medium.

³A vitamin stock solution is made by dissolving 4.88 g thiamine HCl, 2.5 mg biotin, and 2.5 mg B12 in 500 mL deionized water. Adjust to approximately pH 4 before autoclaving 2 min. It is convenient to subdivide the vitamin stock into 10 mL volumes in test tubes prior to autoclaving. Add 10 mL of the vitamin stock solution to each liter of culture medium and 2.5 mL to each liter of test medium.

TABLE 2. SALTS USED IN THE PREPARATION OF GP-2 ARTIFICIAL SEAWATER (30 ‰ SALINITY) FOR USE IN CONJUNCTION WITH NATURAL SEAWATER FOR CHAMPIA CULTURING AND TOXICITY TESTING^{1,2,3,4,5,6}.

Compound	Concentration (g/L)
NaCl	21.03
Na ₂ SO ₄	3.52
KCl	0.61
KBr	0.088
Na ₂ B ₄ O ₇ · 10 H ₂ O	0.034
MgCl ₂ · 6 H ₂ O	9.50
CaCl ₂ · 2 H ₂ O	1.32
SrCl ₂ · 6 H ₂ O	0.02
NaHCO ₃	0.17

¹The original formulation calls for autoclaving anhydrous and hydrated salts separately to avoid precipitation. However, if the sodium bicarbonate is autoclaved separately (dry), all of the other salts can be autoclaved together. Since no nutrients are added until needed, autoclaving is not critical for effluent testing. To minimize microalgal contamination, the artificial seawater should be autoclaved when used for stock cultures. Autoclaving should be for a least 10 min for 1-L volumes, and 20 min for 10-to-20 L volumes.

²Prepare in 10-L to 20-L batches.

³A stock solution of 68 mg/mL sodium bicarbonate is prepared by autoclaving it as a dry powder, and then dissolving it in sterile deionized water. For each liter of GP-2, use 2.5 mL of this stock solution.

⁴From Spotte, et al., 1984.

⁵Effluent salinity adjustment to 30 ‰ can be made by adding the appropriate amount of dry salts from this formulation, by using a triple-strength brine prepared from this formulation, or by using a 100 ‰ salinity brine prepared from natural seawater.

⁶Nutrients listed in Table 1 should be added to the artificial seawater in the same concentration described for natural seawater.

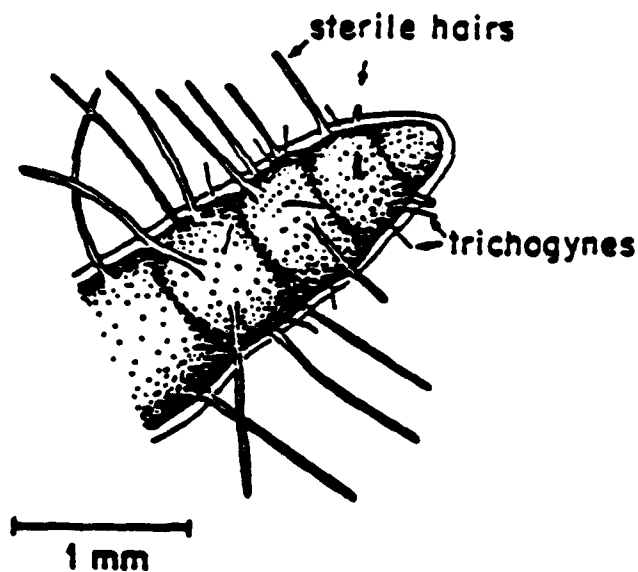


Figure 1. Apex of branch of female plant, showing sterile hairs and reproductive hairs (trichogynes). Sterile hairs are wider and generally much longer than trichogynes, and appear hollow except at the tip. Both types of hairs occur on the entire circumference of the thallus, but are seen easiest at the "edges." Receptive trichogynes occur only near the branch tips. From Thursby and Steele, 1987.

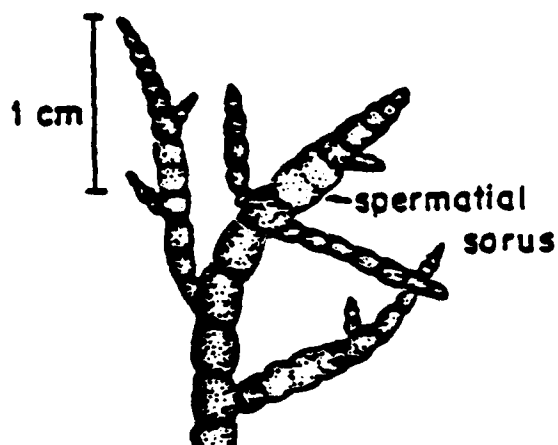


Figure 2. A portion of the male thallus showing spermatial sori. The sorus areas are generally slightly thicker and somewhat lighter in color. From Thursby and Steele, 1987.

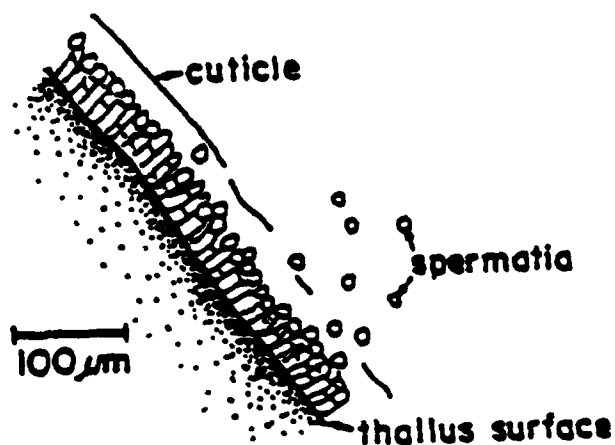


Figure 3. A magnified portion of a spermatial sorus. Note the rows of cells that protrude from the thallus surface. From Thursby and Steele, 1987.

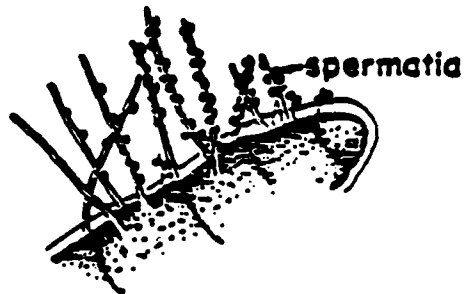


Figure 4. Apex of a branch on a mature female plant that was exposed to spermatia from a male plant. The sterile hairs and trichogynes are covered with spermatia. Note that few or no spermatia attached to the older hairs (those more than 1 mm from the apex). From Thursby and Steele, 1987.

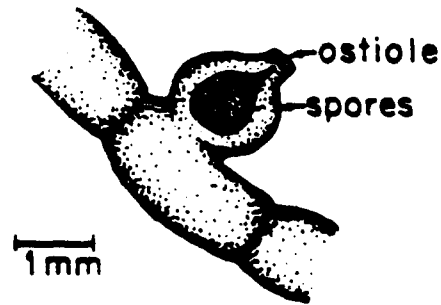


Figure 5. A mature cystocarp. In the controls and lower effluent concentrations, cystocarps often occur in clusters of 10 or 12. From Thursby and Steele, 1987.

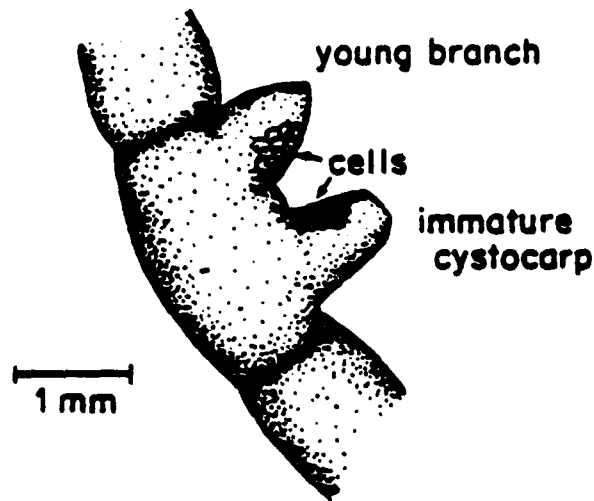


Figure 6. Comparison of a very young branch and an immature cystocarp. Both can have sterile hairs. Trichogynes might or might not be present on a young branch, but are never present on an immature cystocarp. Young branches are more pointed at the apex and are made up of larger cells than immature cystocarps, and never have ostioles. From Thursby and Steele, 1987.

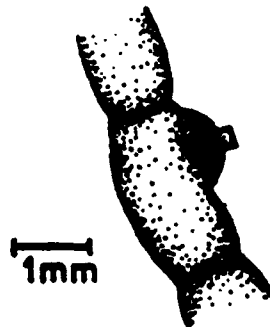


Figure 7. An aborted cystocarp. A new branch will eventually develop at the apex. From Thursby and Steele, 1987.

Figure 2. Data sheet for *Champia parvula* sexual reproduction test.
Cystocarp data sheet.

COLLECTION DATE _____ RECOVERY BEGAN (date) _____

EXPOSURE BEGAN (date) _____ COUNTED (date) _____

EFFLUENT OR TOXICANT _____

TREATMENTS (% EFFLUENT, μ G/L, or REC. WATER SITES)

REPLICATES	CONTROL						
------------	---------	--	--	--	--	--	--

A 1							
2							
3							
4							
MEAN							

B 1							
2							
3							
4							
MEAN							

C 1							
2							
3							
4							
MEAN							

OVERALL MEAN							
-----------------	--	--	--	--	--	--	--

Temperature _____

Salinity _____

Light _____

Source of Dilution Water _____

From Thursby and Steele, 1987.

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING ACUTE TOXICITY TESTS USING
*Ampelisca abdita***

ERL-N SOP 1.03.002
REVISION 0
MAY 1990
PAGE 1 OF 15

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I. OBJECTIVE

To determine the acute toxicity of marine and estuarine sediments to the amphipod *Ampelisca abdita*.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Collection and Holding of Test Organisms

- Boat and accompanying equipment, if the collection site is not otherwise accessible
- Truck, and possibly boat trailer, for transport
- Buckets (and lids for buckets, if sieving is done in the field; this prevents exposure of amphipods to sunlight and ensures containment during transport)
- Waders
- Shoulder-length gloves for winter collections
- Shovel
- Field thermometer
- Vial for collection of sample for salinity (if salinity reading is not to be taken in field)
- Refractometer to measure salinity at collection site
- Miscellaneous personal equipment such as insect repellent, sunscreen, etc. (NOTE: do not allow these materials to come in contact with the amphipods)
- Set of large stainless steel mesh sieves, 2 mm mesh and 0.5 mm mesh
- Large supply of seawater
- Dip nets (fine mesh)
- Glass dishes
- Small diameter (eg. 12 inch) stainless steel mesh sieves, 1.0 mm and 0.5 mm
- Trays for water baths
- Collection permit if necessary

If animals are sieved in the laboratory:

- Fiberglass tank with drain
- Stand for tank
- Blocks, such as bricks or concrete blocks, on which to set sieves
- Apparatus for sieve-shaking
- Apparatus to pour or spray (preferably) seawater on sieve, e.g. buckets, hose and pump, etc.

B. Addition of Sediment to the Test

- Pre-labelled exposure jars and lids
- Labels for excess sediment that may be retained for future testing
- Air and seawater delivery tubes ready to be started
- 2 mm mesh stainless steel sieve*
- Some instrument with which to push sediment through sieve, and container to catch sediment*
- Tool to mix sediment before addition to jars*
- Spoon or spatula for transferring sediment to jars*
- Notebook for recording any notable characteristics of sediments
- Gloves
- Face mask or goggles
- Lab coats
- "Turbulence reducer" = some device to prevent disturbance of the sediment when seawater is added. Two possible designs: A plastic disk with a nylon retrieval line attached (lay flat on sediment surface and then add water), or a glass petri dish with glass tube "handle" glued on with silicone sealant.

*One/treatment will be needed, or a way to clean these items between treatments.
Add clean sediments first.

C. Sorting and Addition of Amphipods

- 0.5 mm stainless steel screen and container to sieve into
- 20°C seawater supply
- Large culture dishes
- Small, fine-mesh dip net
- Containers to sort amphipods into, e.g. 100 ml. plastic specimen cups (clean these and "season" them in seawater for a few days before use to inhibit formation of air bubbles during sorting)
- Glass tube with small bulb for transferring amphipods**
- Dissecting scope and light
- 100 ml. plastic cup with 400-500 um Nitex mesh glued into hole in bottom with silicone sealant**
- Squeeze bottle with 20°C seawater**
- Pieces of solid glass tubing for pushing amphipods under water surface**
- Forceps, probes, petri dish: for removing amphipods from tubes if necessary**
- Scintillation vial and 5% buffered formalin

****Need more than one if more than one person is doing the sorting.**

D. Assay Monitoring

- Thermometer
- Continuous temperature recorder, if possible, to monitor fluctuations in temperature
- Refractometer
- Squeeze bottle with seawater
- Squeeze bottle with deionized water
- Microscope light
- Dissecting microscope
- "Pipette" (glass tube with bulb)
- Data sheets (see Figure 2)
- Solid glass tubing for pushing animals under water surface

For static renewal tests, also:

- Screened siphon tube and "turbulence reducer" (see "Addition of sediments") for daily water changes.

E. Breaking Down the Assay

- 0.5 mm stainless steel sieve
- Container into which contaminated sediment can be sieved
- Final storage container for sediment (pre-disposal)
- Screw-cap jars (about 8 oz.) or specimen cups, one/exposure container
- Squeeze bottles
- Seawater supply
- Pipette (to recover amphipods from water surface)
- Forceps
- 10% buffered formalin with Rose Bengal stain added
- Labels or label tape

F. Examination of Sieved Material for Final Counts

- 0.5 mm sieve
- Container to sieve into
- Waste container for formalin
- Small culture dishes
- Petri dishes
- Forceps
- Probes
- Squeeze bottle with tap water
- Scintillation or other small vials
- 5% buffered formalin
- Dissecting scope

G. Shipping Amphipods

- 500 ml plastic sandwich containers
- Tanks to hold and cap containers
- Cooler(s)
- Ice packs
- Pipettes
- Culture dishes to hold sorted amphipods
- Good light source
- Tape and packing materials

III. METHODS

A. Collection and Holding of Test Organisms

1. Finding a Collection Site for Test Organisms

Ampelisca abdita is a tube-dwelling amphipod found mainly in protected areas from the low intertidal zone to depths of 60 m. It ranges from central Maine to south-central Florida and the eastern Gulf of Mexico (Mills 1964, Bousfield 1973), and has also been introduced into San Francisco Bay (Nichols and Thompson 1985). It has been reported in waters which range from fully marine to 10 parts per thousand salinity (Bousfield 1973). This species generally inhabits sediments from fine sand to mud and silt without shell, although it may also be found in relatively coarser sediments with a sizable fine component (Mills 1967). *A. abdita* is often abundant in sediments with a high organic content (Stickney and Stringer 1957).

A. abdita may be collected throughout the year. However, during certain times of the year, juvenile amphipods may be difficult to obtain. If mature animals are used, adult males must not be tested; they are very active swimmers and they die shortly after mating. A mature male may be identified under a dissecting microscope by its elongated second antennae, which have calceoli attached to the anterior distal margins, and by its carinate muscular urosome (Bousfield 1973).

2. Collecting Amphipods

When a collection site has been located, collection is best done in the morning (to allow for processing time) and at low tide if possible (so that the amphipods will be easier to find and it won't be necessary to go into deep water).

- a. Upon reaching the site, search for patches where *Ampelisca* tubes are numerous--viewing the bottom through a bucket with a plexiglass bottom will help in the search.
- b. Use a shovel to remove the top few inches of sediment and transfer only the uppermost two inches or so into the sieve (if sieved in the field) or buckets (if transported back to laboratory for sieving)-- continue until sufficient numbers of amphipods have been collected.

3. Sieving Amphipods in the Laboratory

Amphipods should be sieved from their native sediment either in the field or as soon as possible (within 24 hours) after collection in the laboratory.

- a. In the laboratory, sieve sediments using large-diameter sieves (a 2 mm mesh sieve nested in a 0.5 mm mesh sieve).
- b. Set sieves on blocks about 5 inches or so (minimum) above the bottom of a containment tank. Processing is easier and less of a physical strain if, in addition, the screens can be suspended from strong, movable boards. The containment tank should have a drain which can be closed off at a level slightly above that of the bottom of the bottom sieve.
- c. Transfer a manageable amount of the sediment containing the amphipods into the top (2 mm) sieve and rinse the < 2 mm material through with a forceful stream of seawater at the collection temperature and salinity. This breaks up the sediment material and also forces most of the animals out of their tubes.
- d. Remove the 2 mm sieve and set aside. Until the sieving and sorting procedures have been completed, save the material retained on the 2 mm screen, which will include a lot of amphipod tubes, in case the tubes need to be reprocessed to obtain more amphipods.
- e. Drain the water level down to about 6 inches above the bottom of the 0.5 mm sieve.
- f. Vigorously shake and swirl the material in the 0.5 mm sieve so that the fine sediments are shaken through and the amphipods are separated from tubes, sediment, and detrital material.
- g. Lift the sieve out of the water and let all the water drain out.
- h. Slowly lower the sieve back into the water. Amphipods will be caught by the surface tension of the water, and can be collected with a fine-mesh dip net.
- i. Transfer the amphipods to large culture dishes or glass baking dishes as sieving continues. The culture dishes of amphipods should be held in a water bath until the next step in processing to maintain them at the collection temperature, and should not be so crowded in a dish that lack of dissolved oxygen becomes a problem.
- j. Further sort amphipods by size using small-diameter 1.0 mm and 0.5 mm sieves.
- k. Sediment collected in the large sieving tank should be allowed to settle

out. The overlying water can then be drained or siphoned out, and the remaining sediment saved for use in holding containers. Some of the fine material will have been lost, but the amphipods will survive.

4. Sieving Amphipods in the Field

- a. Sieve sediment using a 0.5mm mesh screen.
- b. Collect amphipods from the surface tension of the water in the sieve in a manner similar to that described above.
- c. Transport amphipods to the laboratory in covered buckets containing collection site seawater. Care should be taken to avoid exposing the amphipods to sunlight whenever possible.
- d. Further sort amphipods by size using small-diameter 1.0 mm and 0.5 mm sieves.

5. Holding Amphipods in the Laboratory

- a. Amphipod holding jars (1-gallon glass jars containing approximately 3.5-4 cm of sieved collection site sediment) should be set up at least one day before collection to conserve time on collection day and to assure that the sediments will be oxygenated. While seawater is flowing through the holding containers, a screened overflow must be used to prevent loss of swimming amphipods. Approximate density in the holding jars should not exceed 300 amphipods.
- b. Acclimate amphipods from collection temperature to the test temperature of 20°C at no more than 3°C per day.
- c. Amphipods should be used within one week of collection.
- d. During acclimation, amphipods should have food available on a daily basis. During the feeding period (eg. overnight), the flow of seawater to the holding jars should be stopped to increase the exposure of the amphipods to the food source. While the holding jars are in this static condition, jars should be held in a constant temperature chamber or bath to maintain temperature and should be aerated to maintain adequate oxygen concentration and water circulation. Acceptable food sources for *A. abdita* are the diatom Phaeodactylum tricornutum, the golden-brown flagellate Pseudoisochrysis paradoxa, and the diatom Skeletonema costatum (used daily in excess—a suggested amount is 0.5 - 1 liter of dense algal culture per gallon jar).
- e. The procedure for care of amphipods in the laboratory is as follows:
on weekday mornings,

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING ACUTE TOXICITY TESTS USING
*Ampelisca abdita***

ERL-N SOP 1.03.002
REVISION 0
MAY 1990
PAGE 7 OF 15

1. turn off air, connect seawater lines to tubing in each jar
2. check temperature and salinity
3. record observations in notebook

on weekday afternoons, before leaving for the day:

1. connect jar tubing to air lines, hook seawater lines into bucket to form water bath
2. siphon out 1 liter of seawater from each jar through its screened cup
3. check screened cup for any live animals and return them to the jar
4. add 1 liter Phaeodactylum tricornutum to each jar
5. turn on gentle aeration
6. record time and amount fed in notebook
7. record temperature, and if it has been increased (eg. to reach 20°C for a test)

when algae and/or time is short:

1. Feed amphipods at least every 2-3 days and replace the seawater in the holding jars every 2-3 days. Less than one liter of algae may be added to each jar (> or = 500 ml).

B. Addition of Sediment to the Test

CAUTION: Sieving and addition of contaminated sediments should be done in a hood to protect personnel from exposure to volatile contaminants. Addition of sediment takes place one day prior to addition of amphipods.

For each sediment treatment:

1. Press-sieve the sediment through a 2mm stainless steel sieve. This will eliminate very large particles (gravel, etc.) and potential predators (Redmond and Scott 1989).
2. Homogenize sieved sediment thoroughly.
3. Rinse exposure jars with control seawater to wet the inside. The exposure

chamber routinely used to test A. abdita is a quart-sized glass canning jar with a narrow mouth (Figure 1).

4. Spoon a small amount of sediment into each jar. Tap this small amount down to eliminate air pockets. Fill each jar with sediment to 200 ml (about 4 cm.).

5. Add seawater above sediment, using "turbulence reducer" (see supply list) to prevent disturbance of sediment.

6. Hook up exposure jars to air and seawater.

C. Sorting and Addition of Amphipods

1. The water column should be gently aerated with a glass pipette inserted above the sediment surface.

2. Using 0.5 mm sieve, sieve amphipods from holding containers and transfer to large culture dishes.

3. Assemble enough sorting containers (50-100 ml) so that there will be one to correspond to each exposure container plus one extra. Fill each about 3/4 full with control seawater. (NOTE: with a very large experiment, sorting and addition must be done in "sets" of about 10-15 sorting containers each. Ideally there should be an extra sorting container for each set.)

4. Using a pipette, transfer amphipods to the assembled sorting containers. Attempt to select small animals in the same size range. They should all be immature animals, never mature males. Transfer should be done sequentially, i.e. one amphipod in each container, the second in each container, etc.

5. Twenty to thirty amphipods should be tested per replicate. If there are not enough amphipods available, more may be picked from the remaining tubes. However, this procedure is only practical for obtaining a few extra animals since it is very time-consuming.

Procedure for picking additional amphipods from tubes:

1. Under a dissecting scope, hold the end of the tube gently with a pair of forceps and gently move a probe along the tube for a short distance. Move the forceps to this point to prevent the animal from backing up. Then repeat the procedure until the entire tube has been squeezed, and the amphipod will swim out the end unharmed.

6. After all sorting containers are filled, examine each under a dissecting scope and remove any dead or outsized animals and any mature males. Replace these with animals from the sieved population. Recount each group to make sure the correct number of animals is present. Randomize the containers and set one aside (the "extra" one).

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING ACUTE TOXICITY TESTS USING
*Ampelisca abdita***

ERL-N SOP 1.03.002
REVISION 0
MAY 1990
PAGE 9 OF 15

7. Cut off air delivery to exposure jars. For each exposure jar, rinse the contents of one sorting container into a 400- or 500-micron screened cup, and from there into the exposure container. While the amphipods are on the screen, the count can be double-checked since they do not move about much. Be sure that all animals have been transferred, and that none are stuck on container sides.

8. Some amphipods will be floating on the water surface. Using a short piece of solid glass tubing, push them down, making sure that they do not stick on the tubing.

8a. Using a squeeze bottle containing 20°C seawater, rinse down the inner rim and sides of the exposure container down to the water level. Any amphipods stuck to the exposure chamber during addition will be washed down to the water surface. Repeat step 7.

9. Allow amphipods one hour to burrow. Note any not burrowed on data sheet, and then replace those not burrowed with others from the sieved population. (The replacement may be omitted if the absence of burrowing behavior shows a dose-response.)

10. After amphipods are burrowed in, turn air back on.

11. Place screened cup into small culture dish and pour contents of amphipods previously set aside into cup. Float them to the surface. Then using pipette, transfer these animals with a minimal amount of accompanying seawater into a vial containing 5% buffered formalin.

D. Assay Monitoring

1. Record temperature and salinity, other physical measurements if necessary.

2. Remove air and water delivery tubes from exposure jar, checking for any adhering amphipods. Rinse salt out of air pipette ends with squeeze bottle of deionized water.

3. Note any animals floating on the water surface. Rinse down mesh and inside rim of jar with seawater, and note any animals stuck on inner rim. If there are a lot of these in the controls air delivery may be too vigorous.

4. Using a light, examine sediment surface. Record the following:

a. number on water surface and rim of jar

b. number dead

c. number live on sediment surface (out of tubes)

d. number of molts

If the test does not require this much detailed information, "a" and "c" may be combined to number of "emerged" amphipods, and presence or absence, rather than number, of molts may be recorded.

5. If it is uncertain whether or not an animal is dead, remove it with the pipette, place in a small culture or petri dish, and gently prod it with a probe. If the animal does not react, it is dead. Alternatively, if there is a large response to a toxicant, it may be easier and faster to look under a dissecting microscope to determine which animals are dead. Remove any dead animals and molts and discard. (Note: it is a good idea to rinse out "discard" dish after each exposure jar to avoid confusion.)

6. Push under water any animals stuck on the surface.

7. During the first few days of a test, also observe if amphipods have built normal tubes, are actively feeding, and have processed their surrounding sediment.

8. Replace delivery and air tubes.

9. For static renewal tests, after step 6, remove overlying seawater with 400 - 500 micron screened siphon tube to within approximately 1 cm. from sediment surface. Replace with new seawater or appropriate solution, using "turbulence reducer" to prevent disturbing sediment.

E. Breaking Down the Assay

Arrange for collection and disposal of contaminated sediment. (DISPOSAL QUESTIONS SHOULD BE CONSIDERED BEFORE ANY SAMPLES ARE COLLECTED.)

1. Check the assay as usual.

2. For each jar, sieve contents through 0.5mm sieve. (A smaller mesh sieve can be used for the final sieving if there is concern about losing very small animals, but this will make the sieving process more time-consuming.) Sieving may be done with seawater or fresh water, depending on the laboratory situation, and whether or not the sieved material is to be preserved.

3. If the experiment is a small one and personnel are available, the contents of each jar may be rinsed (with seawater, if this is the case) into a culture dish and examined immediately (see description of examination procedure below); live examination is the preferred method for an acute test. However, amphipods and tubes may be rinsed into small screw-cap jars or specimen cups and preserved in 5% buffered formalin (final solution is 5%, made by adding 10% to jars which already contain water) with Rose Bengal stain for later examination.

4. After each sample is sieved, check the sieve to make sure no amphipods are missed. The most effective way to do this is to lower the sieve carefully into the

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING ACUTE TOXICITY TESTS USING
*Ampelisca abdita***

ERL-N SOP 1.03.002
REVISION 0
MAY 1990
PAGE 11 OF 15

sieving water such that any amphipods float to the surface. Also check for tubes stuck to screen and remove with forceps.

F. Examination of Sieved Material for Final Counts

1. With preserved samples, under a hood:

- a. Pour preserved sample into a 0.5mm sieve over some type of waste container (both of these will now be formalin-contaminated).
- b. Rinse sieve with tap water, and transfer rinsed sample into culture dish with enough water so material will float.
- c. Examine material UNDER DISSECTING SCOPE. Remove loose amphipods, sort through shell material or other debris, check all tubes and clusters of material which amphipods may have used as tubes, ie. check everything. Then double check.
- d. Save amphipods recovered in small vials containing 5% buffered formalin. Record number of amphipods recovered. Any amphipods unaccounted for are assumed to be mortalities.
- e. Amphipods which have died in their tubes will generally decompose during the test or break apart during sieving. Occasionally an individual which was dead at the time of test termination will be recovered, and its appearance will be markedly different from those of the amphipods which were alive when preserved. For instance, there may be little tissue within its exoskeleton, it may be contorted, etc. The use of Rose Bengal facilitates recovery of amphipods from the rest of the sieved material. It is not used to separate live from dead individuals. Very recently dead individuals may stain as live, but the number of such animals is assumed to be extremely low. Animals should stain a bright red color. If the staining is spotty, the Rose Bengal may be losing its effectiveness.

2. For fresh animals (not preserved):

- a. The procedure is similar to that for preserved animals, but live animals are separated from dead as in "ASSAY MONITORING".

G. Shipping Amphipods

Ampelisca abdita may be shipped if the time in transit is within one day. Small plastic "sandwich" containers (approximately 500 ml) can be used to hold the amphipods.

1. The containers are filled three-quarters full to a minimum depth of 2 cm of sieved collection site sediment and then to the top with well-aerated seawater.
2. Amphipods are added to the containers with no more than 200 amphipods in each

container.

3. Amphipods should be allowed to burrow into the sediment and build tubes before the containers are capped.
4. The containers must be capped underwater to eliminate any air pockets.
5. Containers should be shipped via overnight delivery in coolers with a few ice packs to prevent temperature fluctuations during transit.
6. Be sure to mark containers "This side up".

IV. TROUBLE SHOOTING

Included in methods section.

V. STATISTICAL ANALYSIS AND DATA USAGE

In tests involving a single compound in seawater only or in spiked sediments, or in tests where a contaminated sediment is diluted with a control sediment and the exposure expressed as percent contaminated sediment, methods for LC50 calculation, such as the trimmed Spearman-Kärber method (Hamilton, Russo, and Thurston, 1977), are used. For other experiments, an arcsine square root transformation of the proportional mortality is conducted before analysis. An analysis of variance (ANOVA) and Duncan's multiple range test (Duncan, 1955) may then be used to compare differences among all treatments, or Dunnett's procedure (Dunnett, 1955) may be used to compare control survival with each of the other treatments.

Data are used to assess the toxicity of contaminated sediment.

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**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING ACUTE TOXICITY TESTS USING
*Ampelisca abdita***

ERL-N SOP 1.03.002
REVISION 0
MAY 1990
PAGE 14 OF 15

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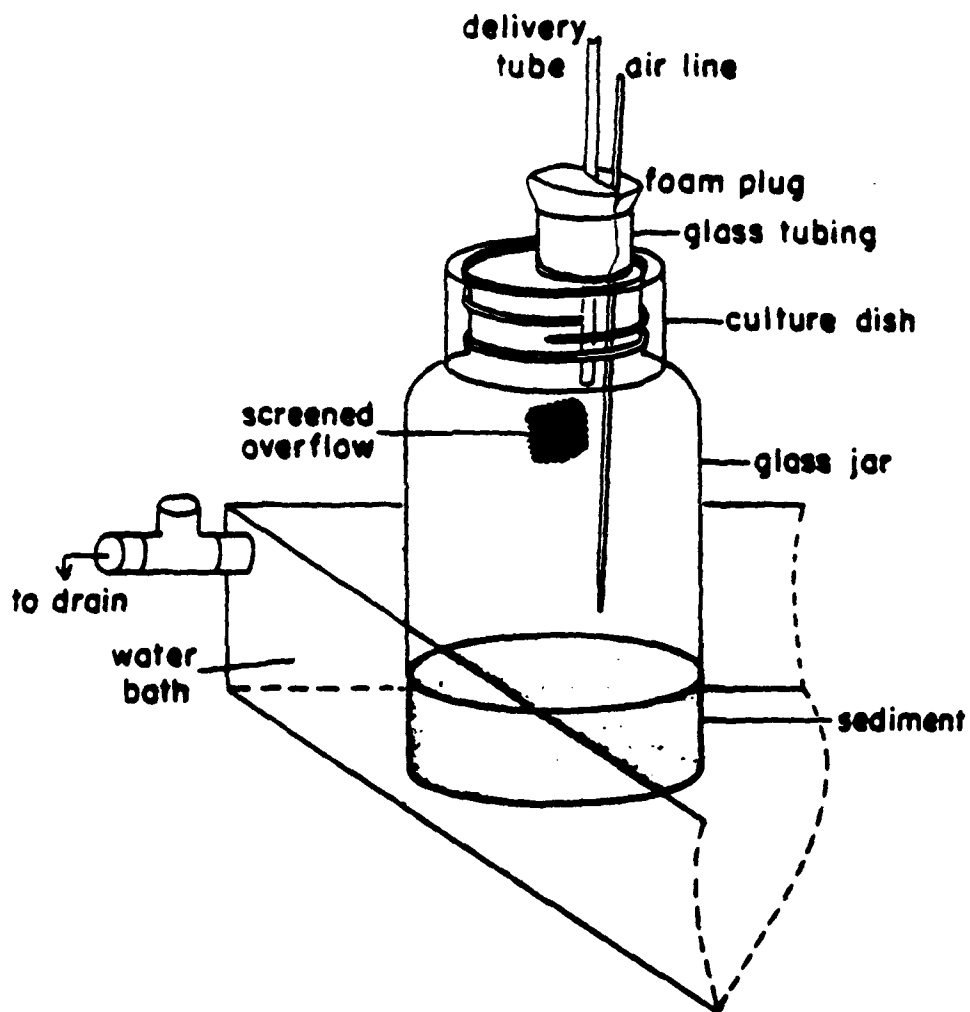


Figure 1 Exposure container used in Aspelisca abdita experiments.

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING ACUTE TOXICITY TESTS USING
Mysidopsis bahia, *Cyprinodon variegatus*, AND *Menidia beryllina***

**ERL-N SOP 1.03.003
REVISION 0
JANUARY 1991
PAGE 1 OF 7**

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I. OBJECTIVE

This standard operating procedure describes static acute effluent toxicity test methods as performed at ERL-N using the species *Mysidopsis bahia*, *Cyprinodon variegatus*, and *Menidia beryllina*.

II. NECESSARY MATERIALS AND EQUIPMENT

- Source of test organisms of known quality
- Environmental chamber or equivalent facility with temperature control--for incubating tests
- Water purification system--deionized water or equivalent
- pH meter--for measuring the pH of effluents
- Dissolved oxygen (DO) meter and BOD probe--for measuring the DO in effluent samples and dilutions
- Refractometer--for determining the salinity of effluent and dilutions
- Thermometers, laboratory grade--for measuring the temperature of effluent samples
- Beakers or Erlenmeyer flasks, glass or non-toxic plasticware--for mixing dilution water
- Wide-bore (4 mm) pipets and bulbs--for distributing test animals
- Water-proof markers--for labelling test vessels
- Colored labelling tape--for labelling test vessels
- Data sheets
- Effluent sample
- Uncontaminated dilution water--natural seawater or hypersaline brine made from natural seawater and diluted with deionized water
- Uncontaminated source of aeration--for aerating effluent and test solutions if necessary
- Exposure chambers--glass or Teflon[®]--pre-soaked in dilution water. Test chamber volumes are indicated in method summaries.
- Plastic sheet--to cover test vessels during test
- Volumetric pipets--Class A, 1-100 mL
- Serological pipets--1-10 mL, graduated
- Pipet fillers--Propipet[®] or equivalent
- Wash bottles--for rinsing equipment
- Bulb-thermograph or electronic-chart type thermometers--for continuously monitoring temperature in water bath or environmental chamber

- Brine shrimp (*Artemia sp.*)--for feeding test organisms
- Laboratory hood--for protection against contamination during preparation of effluent dilutions

III. METHODS

A. Effluent Test Solutions

1. Grab or composite effluent samples should be used within 36 h of the time they are removed from the sampling device.
2. Just prior to testing, the temperature of the effluent should be adjusted to within 1°C of the test temperature (see tables of test conditions for the three test species).
3. The dissolved oxygen (DO) in the effluent should also be checked. If the DO is less than 40% of saturation, the effluent should be aerated before use.
4. The salinity of the effluent should be determined and adjusted to the test salinity (see tables of test conditions for acceptable salinities) using hypersaline brine made from natural seawater (HSB). It is important to check the final effluent salinity using a refractometer.
5. Test dilution water may be natural seawater, receiving water, or HSB diluted with deionized water (DI) (see SOP on brine). If the effluent was salinity adjusted, HSB+DI should be the dilution water and an HSB+DI control should be tested to assure that the HSB was not toxic.
6. Tests include a control and a series of effluent concentrations (usually a minimum of five). The effluent concentrations are commonly selected to approximate a geometric series (eg.-a dilution factor of 0.5). If the effluent is known or suspected to be highly toxic, a range of low effluent concentrations should be used. The solutions are well mixed with a glass rod, TEFLON[®] stir bar or other means. It is important to check the final salinity of all effluent dilutions with a refractometer.

B. Test Organisms

1. Mysids and fish may be obtained from commercial sources or from laboratory cultures (See SOPs on the culture of these test species).

C. Conducting the Tests

1. Appropriate volumes of each effluent concentration are delivered to the test chambers, and the chambers are arranged in random order. The chambers are placed in a water bath or otherwise brought to the required temperature.
2. At least 20 organisms of a given species are exposed to each effluent concentration. Test animals are captured with 4- to 8-mm inside diameter pipettes. Fish larger than 5 mm are captured by dip net. Fish are captured from a common

pool and distributed sequentially to the randomized test chambers until the required number of organisms are placed in each. Mysids are first distributed to small intermediary holding cups (about 10 mL). The entire contents of these cups are then transferred to a test chamber.

3. Two replicates of at least 10 organisms each are provided for each effluent concentration and the control.
4. Test vessels should be covered with a sheet of plastic during the test.
5. The temperature of the test solutions must be maintained within $\pm 1^{\circ}\text{C}$ of the recommended temperature for the test organism (see tables of test conditions). This can be accomplished for static tests by use of a water bath, room air conditioner, or environmental chamber. Temperature in each chamber should be measured at test initiation and daily thereafter.
6. The test vessels are checked for mortality at one, two, four, and eight hours after test initiation and then daily thereafter. Death of fish can be determined by lack of movement (especially gill movement) and no reaction to gentle prodding. Based on mortality results, the following action is taken:
 - a. If the mortality of the organisms in the control test chambers exceeds 10% at any time prior to the scheduled completion of the test, the test has failed to meet the acceptability criterion, and is immediately terminated and repeated. Fresh effluent sample may be required to repeat the test if the holding time has been exceeded.
 - b. If mortality in the effluent exceeds 10% before the scheduled completion of the test, and mortality in the control chambers is less than 10%, the test is continued for the prescribed duration, or until 10% mortality is exceeded in controls, whichever occurs first.
 - c. If, within 1 h of the start of the test, 100% mortality has occurred in the higher effluent concentrations, additional concentrations of effluents should be added to the test at the lower end of the concentration series.
7. In static tests, low DOs commonly occur in the higher concentrations of wastewater. Therefore, it is advisable to monitor the DO closely during the first few hours of the test. Samples with a potential DO problem generally show a downward trend in DO within 4 to 8 h after the test is started. Unless aeration is initiated during the first 8 h of the test, the DO may be exhausted during an unattended period, thereby invalidating the test. Aeration may be accomplished by bubbling air through a 1-mL pipet at a rate of no more than 100 bubbles/min, using an air valve to control the flow. The turbulence caused by aeration should not result in a physical stress to the test organisms. The DO in each chamber with surviving organisms should be monitored daily thereafter.

NOTE: Aeration may alter the results of toxicity tests and should be used

only as a last resort to maintain the required DO. The DO in the test solution must not be permitted to fall below 40% of saturation.

8. Light of the duration, quality, and intensity normally obtained in the laboratory during working hours is adequate (see tables summarizing the test conditions).
9. See tables of test conditions for test durations for the different test species.
10. For the test results to be acceptable, survival in controls must be at least 90%.

IV. TROUBLE SHOOTING

1. A limit is placed on the loading (weight) of organisms per liter of test solution to minimize the depletion of dissolved oxygen, the accumulation of injurious concentrations of metabolic waste products, and/or stress induced by crowding, any of which could significantly affect the test results. However, the probability of exceeding loading limits is greatly reduced with the use of very young test organisms. For both renewal and non-renewal static tests, loading in the test chambers must not exceed 0.4 g/L at 25°C.
2. Minimize stress on test organisms by avoiding unnecessary disturbances.
3. Problems caused by feeding, such as the possible alteration of the toxicant concentration, the build-up of food and metabolic wastes and resulting oxygen demand, are common in static test systems. Where feeding is necessary, excess food should be removed by aspirating with a pipette.

V. STATISTICAL ANALYSIS AND DATA USAGE

Test results are analyzed using the Trimmed Spearman-Kärber method to generate an estimate of the effluent concentration causing 50% mortality (LC50). Data are used to identify discharges which are acutely toxic.

VI. REFERENCES

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**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING ACUTE TOXICITY TESTS USING
Mysidopsis bahia, *Cyprinodon variegatus*, AND *Menidia beryllina***

**ERL-N SOP 1.03.003
REVISION 0
JANUARY 1991
PAGE 5 OF 7**

Table 1. Summary of recommended test conditions and test acceptability criteria for mysid (*Mysidopsis bahia*) acute toxicity tests.

1. Test duration:	96 h
2. Temperature:	20 \pm 2°C
3. Light intensity:	10-20 uE/m ² /s (50-100 ft-c) (ambient laboratory levels)
4. Photoperiod:	16 h light, 8 h darkness
5. Test chamber size:	250 mL
6. Test solution volume:	200 mL
7. Renewal of test concentrations:	Renewed daily (if appropriate)
8. Age of test organisms:	1-5 days, 24 h range in age
9. Organisms per chamber:	10
10. No. replicate chambers per concentration:	2
11. No. organisms per concentration:	20
12. Feeding regime:	Fed two drops of concentrated <i>Artemia</i> nauplii suspension twice daily (approximately 100 nauplii per mysid)
13. Test chamber cleaning:	Cleaning not required
14. Test solution aeration:	None, unless DO concentration falls below 40% saturation; rate should not exceed 100\bubbles/min
15. Dilution water:	25 ‰ \pm 10%
16. Test concentrations:	Minimum of 5 effluent concentrations and a control
17. Dilution series:	0.5
18. Endpoint:	Mortality (LC50)
19. Sample volume required:	4 L (1 gal Cubitainer [®])
20. Test acceptability criterion:	90% or greater survival in controls

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING ACUTE TOXICITY TESTS USING
Mysidopsis bahia, *Cyprinodon variegatus*, AND *Menidia beryllina***

**ERL-N SOP 1.03.003
REVISION 0
JANUARY 1991
PAGE 6 OF 7**

Table 2. Summary of recommended test conditions and test acceptability criteria for sheepshead minnow (*Cyprinodon variegatus*) acute toxicity tests.

1. Test duration:	96 h
2. Temperature:	20 \pm 2°C
3. Light intensity:	10-20 uE/m ² /s (50-100 ft-c) (ambient laboratory levels)
4. Photoperiod:	16 h light, 8 h darkness
5. Test chamber size:	250 mL - 1 L (or modified Norberg-Mount chamber--see SOP on conducting fish larval survival/growth tests)
6. Test solution volume:	200 mL - 750 mL (loading and DO restrictions must be met)
7. Renewal of test concentrations:	Renewed daily (If appropriate)
8. Age of test organisms:	1-14 days, 24 h range in age
9. No. organisms per test chamber:	10
10. No. replicate chambers per concentration:	2
11. No. organisms per concentration:	20
12. Feeding regime:	Food made available during holding prior to used in the test; fed after 48 h, immediately following test solution renewal
13. Test chamber cleaning:	Cleaning not required
14. Test solution aeration:	None, unless DO concentration falls below 40% saturation (3.4 mg/L); rate should not exceed 100\bubbles/min
15. Dilution water:	25 ‰ \pm 10%
16. Test concentrations:	Minimum of 5 effluent concentrations and a control
17. Dilution series:	0.5
18. Endpoint:	Mortality (LC50)
19. Sample volume required:	4 L (1 gal Cubitainer [®])
20. Test acceptability criterion:	90% or greater survival in controls

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING ACUTE TOXICITY TESTS USING
Mysidopsis bahia, *Cyprinodon variegatus*, AND *Menidia beryllina***

**ERL-N SOP 1.03.003
REVISION 0
JANUARY 1991
PAGE 7 OF 7**

Table 3. Summary of recommended test conditions and test acceptability criteria for silversides (*Menidia beryllina*) acute toxicity tests.

1. Test duration:	96 h
2. Temperature:	20 \pm 2°C
3. Light intensity:	10-20 uE/m ² /s (50-100 ft-c) (ambient laboratory levels)
4. Photoperiod:	16 h light, 8 h darkness.
5. Test chamber size:	250 mL - 1 L (or modified Norberg-Mount chamber--see SOP on conducting fish larval survival/growth tests)
6. Test solution volume:	200 - 750 mL; loading and DO restrictions must be met
7. Renewal of test concentrations:	Renewed daily (If appropriate)
8. Age of test organisms:	7-14 days; 24 h range in age
9. No. organisms per test chamber:	10
10. No. replicate chambers per concentration:	2
11. No. organisms per concentration:	20
12. Feeding regime:	Food made available during holding period prior to use in the test; fed after 48 h, immediately following test solution renewal
13. Test chamber cleaning:	Cleaning not required
14. Test solution aeration:	None, unless DO concentration falls below 40% saturation; rate should not exceed 100\bubbles/min
15. Dilution water:	25 ‰ \pm 10%
16. Test concentrations:	Minimum of 5 effluent concentrations and a control
17. Dilution series:	0.5
18. Endpoint:	Mortality (LC50)
19. Sample volume required:	4 L (1 gal Cubitainer [®])
20. Test acceptability criterion:	90% or greater survival in controls

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE INLAND SILVERSIDE (*Menidia beryllina*)
AND SHEEPSHEAD MINNOW (*Cyprinodon variegatus*)
LARVAL SURVIVAL AND GROWTH TESTS**

ERL-N SOP 1.03.004
REVISION 0
JANUARY 1991
PAGE 1 OF 17

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I. OBJECTIVE

This method estimates the chronic toxicity of effluents and receiving waters to inland silverside (*Menidia beryllina*) or sheepshead minnow (*Cyprinodon variegatus*) larvae in a seven-day, static-renewal test.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Construction of Modified Norberg-Mount chambers (Figure 1)

- 1/8 inch thick plates of glass
- Ruler
- All-surface marker
- Glass cutter
- Wet belt sander -- to sand the edges of cut glass pieces
- Heavy gloves and safety glasses -- for protection from unsanded glass and glass shards
- 200 um mesh nylon screen -- to separate main chamber from sump area
- Silicone cement -- to cement glass sections together and attach screen
- Large metal 'paper clamps' -- to hold screen and glass supports in place while silicone cement sets

NOTE: 1000 mL glass beakers may also be used as test chambers.

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE INLAND SILVERSIDE (*Menidia beryllina*)
AND SHEEPSHEAD MINNOW (*Cyprinodon variegatus*)
LARVAL SURVIVAL AND GROWTH TESTS**

ERL-N SOP 1.03.004
REVISION 0
JANUARY 1991
PAGE 2 OF 17

B. Conducting the Test

- Facilities for holding and acclimating test organisms.
- *Artemia* nauplii, newly hatched -- for food during test -- see SOP on Culture.
- Environmental chamber or equivalent facility with temperature control ($25 \pm 2^{\circ}\text{C}$).
- Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.
- Balance, analytical -- capable of accurately weighing to 0.0001 g.
- Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing boats and the expected weights of the weighing boats plus fish.
- Drying oven -- 60°C , for drying larvae.
- Air pump -- for oil free air supply.
- Air lines, plastic or pasteur pipettes, or air stones -- for gently aerating water containing the fragile larvae and or supplying air to test solution with low DO.
- pH and DO meters and probes -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.
- Desiccator -- for holding dried larvae.
- Light box -- for counting and observing larvae.
- Refractometer -- for determining salinity.
- Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.
- Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.
- Plastic sheets -- for covering chambers during test.
- Beakers -- at least six Class A, borosilicate glass or non-toxic plasticware, 4000 mL for making test solutions.
- Wash bottles -- for deionized water and seawater, for rinsing small glassware and instrument electrodes and probes.
- Crystallization dishes, beakers, culture dishes, or equivalent -- for holding larvae during distribution for a test.
- Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.
- Pipets, volumetric -- Class A, 1-100 mL.
- Pipets, automatic -- adjustable, 1-100 mL.
- Pipets, serological -- 1-10 mL, graduated.
- Pipet bulbs and fillers -- PROPIPET[®], or equivalent.
- Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring larvae.
- Siphon with bulb and clamp -- for cleaning test chambers.
- Forceps -- for transferring dead larvae to weighing boats.
- NITEX[®] mesh sieves ($\leq 150 \mu\text{m}$ and $500 \mu\text{m}$) -- for collecting *Artemia* nauplii and fish larvae.
- Data sheets (one set per test) -- for data recording (Figure 2).
- Tape, colored -- for labelling test chambers
- Markers, water-proof -- for marking containers, etc.
- Weighing boats, aluminum -- 20/test (2 extra).
- pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for

ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE INLAND SILVERSIDE (*Menidia beryllina*)
AND SHEEPSHEAD MINNOW (*Cyprinodon variegatus*)
LARVAL SURVIVAL AND GROWTH TESTS

ERL-N SOP 1.03.004
REVISION 0
JANUARY 1991
PAGE 3 OF 17

standards and calibration check.

- Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.
- Effluent, surface water, and dilution water.
- 7-to-11-day-old inland silversides larvae or <24-hr-old sheepshead minnow larvae -
- This test requires from 180 to 360 larvae, obtainable from a laboratory culture or from a commercial source (see SOP on Culture).

III. METHOD

A. Construction of a Modified Norberg-Mount Chamber

1. Cut two end, two side, 1 bottom, and 3 sump support pieces from the plate glass to the sizes shown in Figure 1. NOTE: Be sure to wear gloves and safety glasses while handling unsanded glass.
2. Using a belt wet-sander, sand the edges and corners of the pieces of glass to eliminate all sharp edges. NOTE: Be sure to wear safety glasses.
3. Wash and thoroughly dry the glass.
4. Apply a thin silicone bead to two adjacent edges of one side piece and carefully set aside without disturbing the silicone. Immediately apply a thin silicone bead to one edge of one end piece. Avoid excessive use of silicone on the interior of the chamber, as this material may tend to collect some organic compounds which may affect test results. However, enough silicone should be used to ensure that the chambers do not leak and the larvae cannot get trapped or escape into the sump area.
5. Carefully place the silicone edge of the side piece on top of the bottom piece flush with the edge of the bottom piece and hold in place. Carefully set the silicone edge of the end piece on the bottom piece flush with one end of the bottom piece and press against the silicone on the upright edge of the side piece, forming an 'L' shape on the bottom piece. Release the pieces (they should be 'glued' together well enough to stand alone) and allow the silicone to set for at least 2 hours.
6. Cut a piece of 200 um nitex 8 cm wide and about 12 cm long. Apply a thick bead of silicone to the face of one of the 8 cm sump support pieces. 'Sandwich' one end of the nitex between the silicone side of the support piece and the side of the chamber about one inch from the open end of the chamber with the nitex mesh inside the chamber (there should be little or no nitex protruding from under the support piece toward the open end of the chamber). Clamp the 'sandwich' and allow to dry for at least 12 hours.
7. This step can be performed immediately after step 6. Apply a thin silicone bead to two adjacent sides of the other side piece. Carefully set the silicone long edge on the bottom piece and press the silicone short edge against the end piece, flush with the edge of the end piece and the edge of the bottom piece forming a 'U' shape on the bottom piece. Avoid disturbing the clamped nitex and support.

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE INLAND SILVERSIDE (*Menidia beryllina*)
AND SHEEPSHEAD MINNOW (*Cyprinodon variegatus*)
LARVAL SURVIVAL AND GROWTH TESTS**

ERL-N SOP 1.03.004
REVISION 0
JANUARY 1991
PAGE 4 OF 17

8. Once the silicone from steps 6 and 7 has dried, apply a thick silicone bead to the other 8 cm sump support. Pull the nitex as tightly as possible against the opposite side of the chamber and tightly press the support against the nitex and side of the chamber, forming a screen parallel to and one inch away from the open end of the chamber. Remove the clamp from the first 'sandwich' and tightly clamp the second. Allow to set for at least 12 hours.

9. After the silicone from step 8 has dried, apply thin silicone beads to one long edge and one face of the remaining sump support piece and press against the bottom of the chamber at the bottom of the screen. Press the screen against the silicone on the face of the support. Also, using as little silicone as possible, seal the bottom and ends of the support in the sump area (the main part of the chamber should be able to hold water about 1 cm deep without leaking into the sump area).

10. When the silicone has set (after about 2 hours), apply two thin silicone beads in the main part of the chamber--one in each crack between the nitex screen and the glass sides of the chamber. This prevents very tiny larvae from getting stuck in these areas.

11. Immediately after step 10, apply a thin silicone bead to one edge of the remaining end piece and the short edges of the two sides. Carefully press the end against the bottom and sides of the chamber and allow to set for at least 12 hours.

12. Once constructed, check the chambers for leaks and repair if necessary (any extra silicone should be applied on the outside of the chamber). Soak the chambers overnight in sea water (preferably in flowing water) to cure the silicone cement before use. Chambers should be also be soaked in seawater overnight after they have been cleaned.

B. Conducting the Test

1. Test Solutions

a. Surface water toxicity is determined with samples used directly as collected as long as the salinity of the sample is between 5 and 32 ‰ for inland silverside tests or between 20 and 30 ‰ for sheepshead minnow tests. If salinity adjustment is necessary, this is accomplished using hypersaline brine (see SOP on Brine) and a brine + deionized water control of the same salinity must be tested. Natural seawater and/or uncontaminated local water may be tested as additional controls.

b. The selection of the effluent test concentrations should be based on the objectives of the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. If hypersaline brine is used to adjust salinities, the maximum effluent concentration will be 80% at 20 ‰ salinity, and 70% at 30 ‰ salinity. If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used, beginning at 10%. If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions at the lower range (3%, 1%, 0.3%, and 0.1%) of effluent concentrations should be added. About 2500 mL of each solution are needed to start/renew a test.

ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE INLAND SILVERSIDE (*Menidia beryllina*)
AND SHEEPSHEAD MINNOW (*Cyprinodon variegatus*)
LARVAL SURVIVAL AND GROWTH TESTS

ERL-N SOP 1.03.004
REVISION 0
JANUARY 1991
PAGE 5 OF 17

c. Tests should begin as soon as possible after sample collection, preferably within 24 h. If the persistence of the sample toxicity is not known, the maximum holding time should not exceed 36 h for off-site toxicity studies. Samples should be stored at 4°C at all times prior to use. Just prior to testing, the temperature of the sample should be adjusted to $(25 \pm 2^{\circ}\text{C})$ and maintained at that temperature until portions are added to the dilution water.

2. Starting the Test

a. One day prior to beginning of test, set up the *Artemia* culture so that newly hatched nauplii will be available on the day the test begins (see SOP on culture).

b. Increase the temperature of water bath, room, or incubator to the required test temperature $(25 \pm 2^{\circ}\text{C})$.

c. Label the test chambers with colored tape and a marking pen to identify each concentration and replicate.

d. *M. beryllina* larvae seven to 11 days old can be used to start the survival and growth test. At 25°C, this test can be performed at salinities of 5 ‰ to 32 ‰. If the specific salinity required for the test differs from the rearing salinity, adjustments of 5 ‰ daily should be made over the three days prior to start of test.

e. *C. variegatus* larvae less than 24 hr old can be used to start a survival and growth test. If embryos have been incubating at 25°C and 30 ‰ salinity for five-to-six days, approximately 24 h prior to hatching the salinity of the sea water in the incubation chamber may be reduced from 30 ‰ to the test salinity, if lower than 30 ‰. A few larvae may hatch 24 h ahead of the majority. Remove these larvae and reserve them in a separate dish, maintaining the same culture conditions. It is preferable to use only the larvae that hatch in the 24 h prior to starting the test. However, if sufficient numbers of larvae do not hatch within the 24-h period, the larvae that hatch prior to 24 h are added to the test organisms. The test organisms are then randomly selected for the test.

f. Gently remove larvae by concentrating them in a corner of the aquarium or culture dish, and capturing them with a wide-bore pipette, small crystallization dish, (3 - 4 cm in diameter), or small beaker. Transfer larvae 1 - 3 at a time using a wide bore pipet into each test chamber (chambers should be randomly arranged) in sequential order until the desired number (10-15) is attained. After the larvae are dispensed, place the chambers on a light table and verify the number of larvae in each chamber.

NOTE: Because *M. beryllina* larvae are very sensitive to handling, it is advisable to distribute them to their respective test chambers which contain control water on the day before the test is to begin. At the start of the test, remove any dead or unhealthy larvae and replace with fresh larvae.

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE INLAND SILVERSIDE (*Menidia beryllina*)
AND SHEEPSHEAD MINNOW (*Cyprinodon variegatus*)
LARVAL SURVIVAL AND GROWTH TESTS**

ERL-N SOP 1.03.004
REVISION 0
JANUARY 1991
PAGE 6 OF 17

g. The test is started by removing most of the clean sea water from each test chamber (leaving about 1 cm of seawater in the bottom of the chamber so the larvae will not be stranded) and replacing with 500-750 mL of the appropriate test solution.

h. Randomize the position of test chambers at the beginning of the test, using a position chart. Assign numbers for the position of each test chamber using a table of random numbers or similar process. Maintain the chambers in this configuration throughout the test.

3. Test Conditions

a. The light quality and intensity should be at ambient laboratory levels, which is approximately 10 - 20 uE/m²/s, or 50 to 100 foot candles (ft-C), with a photoperiod of 14 h of light and 10 h of darkness.

b. The test salinity should be in the range of 5 ‰ to 32 ‰ for inland silverside tests and 20 ‰ to 30 ‰ for sheepshead minnow tests. The salinity should not vary by more than ± 2 ‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

c. The water temperature in the test chambers should be maintained at $25 \pm 2^{\circ}\text{C}$.

d. Dissolved oxygen in the test chambers should not fall below 60% of saturation. The DO should be measured on new solutions at the start of the test (Day 0). If it is necessary to aerate, all concentrations and the control should be aerated. The aeration rate should not exceed 100 bubbles/min, using a pipet with an orifice of approximately 1.5 mm, such as a 1-mL, Kimax serological pipet, No. 37033, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue stress to the fish. Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO.

4. Feeding

a. The test larvae are fed newly-hatched (less than 24-h-old) *Artemia* nauplii once a day from Day 0 through Day 6; larvae are not fed on Day 7. Sufficient numbers of nauplii should be fed to ensure that some remain alive overnight in the test chambers. An adequate, but not excessive amount, should be provided to each replicate on a daily basis. Feeding excessive amounts of nauplii will result in a depletion in DO to below an acceptable level. As much of the uneaten *Artemia* as possible should be siphoned from each chamber daily to ensure that the larvae principally eat newly hatched nauplii.

b. On days 0, 1, and 2, transfer 4 mL of concentrated, rinsed nauplii to about 50 mL of clean seawater in a 100 mL beaker, and bring to a volume of 80 mL

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE INLAND SILVERSIDE (*Menidia beryllina*)
AND SHEEPSHEAD MINNOW (*Cyprinodon variegatus*)
LARVAL SURVIVAL AND GROWTH TESTS**

ERL-N SOP 1.03.004
REVISION 0
JANUARY 1991
PAGE 7 OF 17

with more seawater.

c. Aerate or swirl the suspension to equally distribute the nauplii while withdrawing individual 2 mL portions of the nauplii suspension by pipette or adjustable syringe to transfer to each replicate test chamber. Because the nauplii will settle and concentrate at the tip of the pipette during the transfer, limit the volume of concentrate withdrawn each time to a 2-mL portion for one test chamber helps ensure an equal distribution to the replicate chambers. Equal distribution of food to the replicates is critical for successful tests.

d. On Days 3-6, transfer 6 mL of concentrated nauplii to about 50 mL seawater in a 100 mL beaker. Bring to a volume of 80 mL with more seawater and dispense as described above.

e. If the larvae survival rate in any replicate on any day falls below 50%, reduce the volume of *Artemia* nauplii suspension added to that test chamber by one-half (i.e., reduce from 2 mL to 1 mL) and continue feeding one-half the volume through Day 6.

f. Record the time of feeding on the data sheets.

5. Daily Monitoring of the Test

a. Approximately 1 h before test initiation, a sufficient quantity of effluent or receiving water sample is warmed to $25 \pm 2^{\circ}\text{C}$ to prepare the test solutions.

b. DO is measured at the end of each 24-h exposure period (just before test chamber cleaning and renewal) in one test chamber at all test concentrations and in the control.

c. Temperature, pH, and salinity are measured at the end of each 24-h exposure period in one test chamber at all test concentrations and in the control. The pH is measured in the effluent sample each day.

d. The number of live larvae in each test chamber are recorded daily and the dead larvae are discarded.

e. Before the daily renewal of test solutions, uneaten and dead brine shrimp, and other debris are removed from the bottom of the test chambers with a siphon hose. Alternately, a large pipet (50 mL), fitted with a safety pipet filler or rubber bulb, can be used. If the test chambers illustrated in Figure 1 are used, remove only as much of the test solution from the chamber as is necessary to clean, and siphon the remainder of the test solution from the sump area. Because of their small size during the first few days of the test, larvae are easily drawn into a siphon tube when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of larvae can be greatly reduced because they can be more easily seen. If the water siphoned from the test chambers is collected in a white plastic tray, the larvae caught up in the siphon can be

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE INLAND SILVERSIDE (*Menidia beryllina*)
AND SHEEPSHEAD MINNOW (*Cyprinodon variegatus*)
LARVAL SURVIVAL AND GROWTH TESTS**

ERL-N SOP 1.03.004
REVISION 0
JANUARY 1991
PAGE 8 OF 17

carefully retrieved and gently returned by pipette to the appropriate test chamber. When handling mortality occurs, record the number and chamber position of dead larvae.

f. The test solutions are renewed daily, immediately after cleaning the test chambers. The water level in each chamber is lowered to a depth of 7-to-10 mm, leaving 10 to 15% of the test solution. New test solution is added slowly by refilling each chamber with the appropriate amount of test solution without excessively disturbing the larvae. If the modified Norberg-Mount chamber is used, renewals should be poured into the sump area using a narrow bore (approximately 9 mm ID) funnel.

NOTE: Daily test observations, solution renewals, and removal of dead larvae, should be carried out carefully to protect the larvae from unnecessary disturbance during the test. Care should be taken to see that the larvae remain immersed at all times during the performance of the above operations.

6. Termination of the Test

a. At termination (Day 7), the number of surviving larvae in each test chamber are counted.

b. Pour all live larvae from a replicate onto a 500 um mesh screen in a large beaker to retain the larvae and allow *Artemia* to be rinsed away. Rinse the larvae in an ice bath of deionized water to remove salts that might contribute to the dry weight--this also immobilizes the larvae.

c. Using fine-tipped forceps, carefully place the larvae on a small pre-weighed aluminum weighing boat for drying and weighing. An appropriate number of these aluminum weigh boats (one per replicate) are marked for identification and pre-weighed to 0.01 mg, and the weights are recorded on the data sheets.

d. The larvae are dried at 60°C for 24 h.

e. Immediately upon removal from the drying oven, the weighing boats are placed in a desiccator to cool and to prevent the adsorption of moisture from the air until weighed. Weigh all weighing boats containing the dried larvae to 0.01 mg, subtract the tare weight to determine dry weight of larvae in each replicate, and record on the data sheets.

f. Divide the dry weight by the number of larvae per replicate to determine the average dry weight, and record on the data sheets. Complete the summary data sheet after calculating the average measurements and statistically analyzing the dry weights and per cent survival for the entire test.

g. *Menidia beryllina* test results are acceptable if (1) the average survival of control larvae is equal to or greater than 80%, and (2) where the test starts with 7-day old larvae, the average dry weight of the control larvae is equal to or

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE INLAND SILVERSIDE (*Menidia beryllina*)
AND SHEEPSHEAD MINNOW (*Cyprinodon variegatus*)
LARVAL SURVIVAL AND GROWTH TESTS**

ERL-N SOP 1.03.004
REVISION 0
JANUARY 1991
PAGE 9 OF 17

greater than 0.50 mg. *Cyprinodon variegatus* test results are acceptable if (1) the average survival of control larvae is equal to or greater than 80%, and (2) where the test starts with larvae less than 24 hr old, the average dry weight of the control larvae is equal to or greater than 0.60 mg.

IV. TROUBLE SHOOTING

1. Toxic substances may be introduced by contaminants in glassware and testing equipment. Make sure that all pieces of equipment are thoroughly clean and/or have been soaked in clean seawater.
2. Highly degradable or volatile compounds may be lost from the open test chambers. Therefore, chambers should be covered with plastic sheeting while the test is in progress.
3. Food added during the test may sequester metals and other toxic substances and confound test results. Therefore, excessive feeding should be avoided.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data.
2. The end points of toxicity tests using the inland silverside are based on the adverse effects on survival and growth. Least effect and no effect concentrations (LOECs and NOECs) for survival and growth are obtained by one-way analysis of variance (ANOVA) followed by Dunnett's Procedure (Dunnett, 1955) for comparing several treatments to a control.
3. Data are used, along with data from other toxicity tests, to assess the toxicity of effluents and receiving waters. Data may also be used in the NPDES permit process.

VI. REFERENCES

- Dunnett, C.W. 1955. A multiple comparisons procedure for comparing several treatments with a control. JASA 50:1096-1101.
- US EPA. 1988. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. Weber, C.I., et al (eds). EPA Office of Research and Development EPA-600/4-87/028 (May 1988).

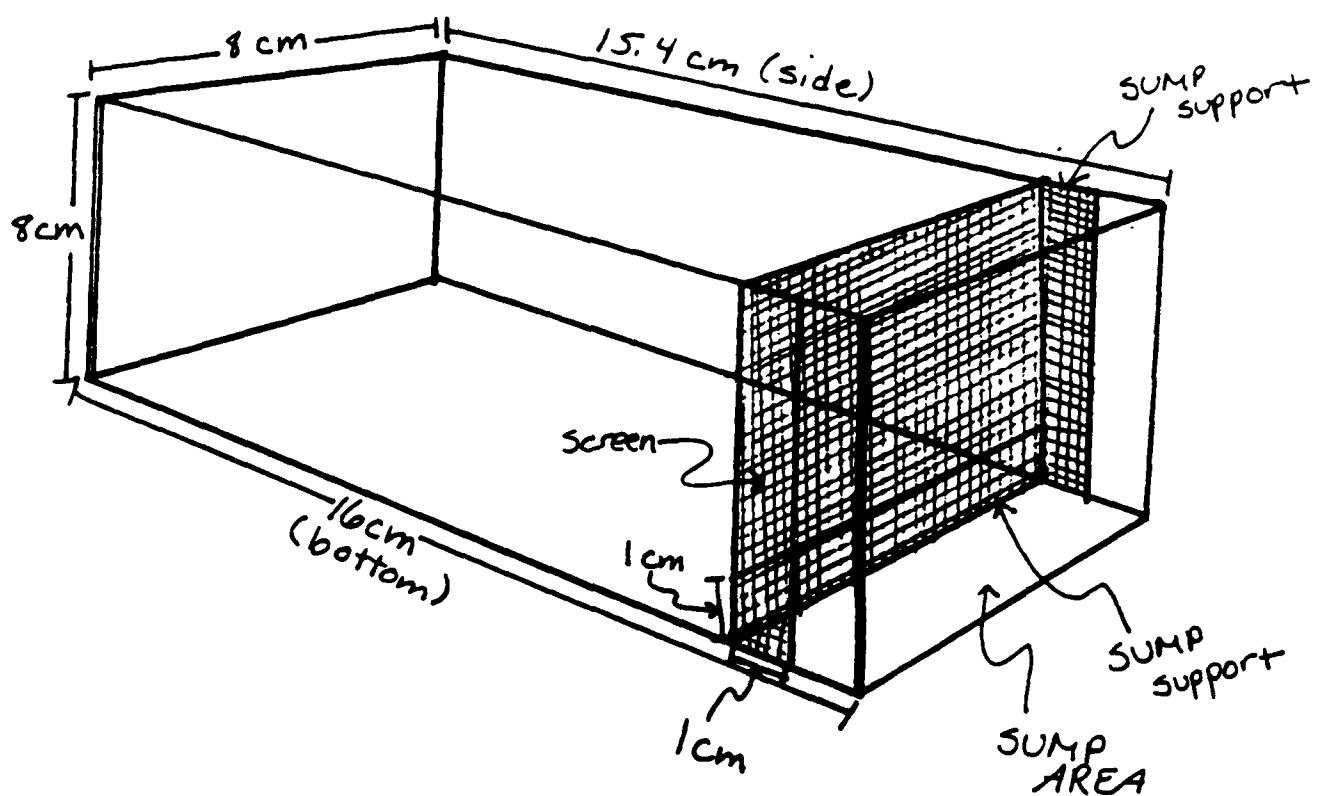


Figure 1. Modified Norberg-Mount chamber.

FIGURE 2. Sample data sheet

Fish Growth/Survival Study

Test Dates: _____
 Date of Hatch: _____
 Type of Effluent: _____
 Effluent Tested: _____

Species: _____
 Field: _____ Lab: _____ Test: _____

Treatment: Control									
Rep: a	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: b	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: c	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Comments:

Treatment:									
Rep: a	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: b	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: c	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Day	0	1	2	3	4	5	6
Time Fed							
Amt. Fed							

Test Dates: _____

Species: _____

Effluent Tested: _____

Treatment:									
Rep: a	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: b	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: c	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Comments:

Treatment:									
Rep: a	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: b	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: c	Pan#:				Pan wt.:				
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Test Dates: _____

Species: _____

Effluent Tested: _____

Treatment:									
Rep: a	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: b	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: c	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Comments:

Treatment:									
Rep: a	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: b	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: c	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Test Dates: _____

Species: _____

Effluent Tested: _____

Treatment:									
Rep: a	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: b	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: c	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Comments:

Treatment:									
Rep: a	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: b	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								
Rep: c	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Test Dates: _____

Species: _____

Effluent Tested: _____

Treatment:									
Rep: a	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Treatment:									
Rep: b	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Treatment:									
Rep: c	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Treatment:									
Rep: a	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Treatment:									
Rep: b	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Treatment:									
Rep: c	Pan#:								Pan wt.:
Day	0	1	2	3	4	5	6	7	
Live									
Temp. (°C)									
Salinity (‰)									
D.O. (mg/l)									
# Fish:	Wt., Fish + Pan:								

Comments: _____

Test Dates: _____ Species: _____

Effluent Tested: _____

Treatment						
Number Live						
Percent Live						
Ave. Wt./ Larvae (mg)						
Sig. Diff. from Ctl.						
Average Temp. (°C)						
Average Sal. (‰)						
Average D.O. (mg/l)						

Comments:

Test Dates: _____ Species: _____

Effluent Tested: _____

Treatment						
Number Live						
Percent Live						
Ave. Wt./ Larvae (mg)						
Sig. Diff. from Ctl.						
Average Temp. (°C)						
Average Sal. (‰)						
Average D.O. (mg/l)						

Comments:

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE *Mysidopsis bahia* SURVIVAL, GROWTH,
AND FECUNDITY TEST**

ERL-N SOP 1.03.005
REVISION 0
JANUARY 1991
PAGE 1 OF 13

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I. OBJECTIVE

This method estimates the chronic toxicity of effluents and receiving waters to the estuarine mysid, *Mysidopsis bahia*, during a seven-day, static-renewal exposure.

II. NECESSARY MATERIALS AND EQUIPMENT

- 7-day-old juvenile mysids -- a minimum of 240, obtainable from laboratory cultures or from a commercial supplier (see SOP on Culture).
- Facilities for holding and acclimating test organisms -- See SOP on Culture.
- *Artemia* nauplii, newly hatched -- see SOP on Culture.
- Environmental chamber or equivalent facility with temperature control (26 -27°C).
- Water purification system -- Millipore Super-Q, deionized water or equivalent.
- Balance -- capable of accurately weighing to 0.000001 g.
- Reference weights, Class S -- for checking performance of balance. Reference weights should bracket the expected weights of the weighing boats and weighing boats plus organisms.
- Drying oven -- 60°C, for drying organisms.
- Desiccator -- for holding dried organisms.
- Air pump -- for supplying air.
- Air lines, and air stones -- for aerating cultures, brood chambers, and holding tanks, and supplying air to test solutions with low DO.
- pH and DO meters -- for routine physical and chemical measurements.
- Tray -- for test vessels; approximately 90 X 48 cm to hold 48 vessels.
- Standard or micro-Winkler apparatus -- for determining DO and checking DO meters.
- Dissecting microscope (350-400X magnification) -- for examining organisms in the test vessels to determine their sex and to check for the presence of eggs in the oviducts of females.
- Light box -- for illuminating organisms during examination.
- Refractometer -- for determining salinity.

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE *Mysidopsis bahia* SURVIVAL, GROWTH,
AND FECUNDITY TEST**

ERL-N SOP 1.03.005
REVISION 0
JANUARY 1991
PAGE 2 OF 13

- Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.
- Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.
- Test vessels -- 200 mL borosilicate glass beakers or 8 oz disposable plastic cups (manufactured by Falcon Division of Becton, Dickinson Co., 1950 Williams Dr., Oxnard, CA 93030) or other similar containers. Cups must be soaked overnight in dilution water and then rinsed in deionized water before use. Forty-eight (48) test vessels are required for each test (eight replicates at each of five effluent concentrations and a control).
- Beakers or flasks -- six, borosilicate glass or non-toxic plasticware, 2000 mL for making test solutions.
- Wash bottles -- containing deionized water and clean seawater, for washing organisms from containers and for rinsing all glassware and instrument electrodes and probes.
- Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 50-2000 mL, for making test solutions.
- Separatory funnels, 2-L -- Two-four for culturing *Artemia*.
- Pipets, volumetric -- Class A, 1-100 mL.
- Pipets, automatic -- adjustable, 1-100 mL.
- Pipets, serological -- 1-10 mL, graduated.
- Pipet bulbs and fillers -- PROPIPET[®], or equivalent.
- Droppers, and glass tubing with fire polished edges, 4 mm ID -- for transferring organisms.
- Forceps -- for transferring organisms to weighing boats.
- NITEX[®] mesh sieves (150 μ m and 1000 μ m) -- for concentrating organisms.
- Depression glass slides or depression spot plates -- two, for observing organisms.
- Data sheets (one set per test) -- for data recording (Figure 1).
- Tape, colored and markers, water-proof -- for labelling and marking test chambers, containers, etc.
- Weighing boats, aluminum -- to determine the dry weight of organisms.
- Buffers, pH 4, 7, and 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check.
- Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.
- Effluent, surface water, and dilution water.

III. METHODS

A. Test Solutions

1. Surface water toxicity is determined with samples used directly as collected when the sample salinity is 20 to 30 ppt. If salinity adjustment is necessary, this is accomplished using hypersaline brine (see SOP on Brine) and a brine + deionized water control of the same salinity must be tested. Natural seawater and/or uncontaminated local water may be tested as additional controls.
2. The selection of the effluent test concentrations should be based on the objectives of

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE *Mysidopsis bahia* SURVIVAL, GROWTH,
AND FECUNDITY TEST**

ERL-N SOP 1.03.005
REVISION 0
JANUARY 1991
PAGE 3 OF 13

the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used, beginning at about 10%. If high mortality is observed during the first 1 or 2 h of the test, additional lower effluent concentrations can be added.

3. The volume of effluent required for daily renewal of eight replicates per concentration, each containing 150 mL of test solution, is approximately 1200 mL. Prepare enough test solution (approximately 1.5 L).

4. The test should begin as soon as possible after sample collection, preferably within 24 h. If the persistence of the sample toxicity is not known, the maximum holding time should not exceed 36 h for off-site toxicity studies. Samples should be stored at 4°C at all times prior to use.

B. Starting the Test

1. About 48 hr prior to beginning the test, set up the *Artemia* culture so that nauplii will be available on the day the test begins (see SOP on Culture).

2. Increase the temperature of the water bath, room, or incubator to the required test temperature (26 - 27°C).

3. About 1 h before test initiation, the temperature of the sample should be adjusted to the test temperature (26 - 27°C) and maintained at that temperature while the dilutions are being made. Effluent dilutions should be prepared for all replicates in each treatment in one flask to ensure low variability among the replicates. The salinity and temperature of each dilution should be checked prior to addition to test chambers.

4. Label the test chambers (cups) with colored tape and a marking pen to identify each concentration and replicate number.

5. Dispense 150 mL of the appropriate effluent dilution to each cup.

6. Randomly place five 7-day-old animals (one at a time) in each test cup of each treatment using a large bore (4 mm ID) pipette. It is easier to capture the animals if the volume of water in the dish holding the test animals is reduced and the dish is placed on a light table. It is recommended that the transfer pipette be rinsed frequently because mysids tend to adhere to the inside surface.

7. Randomize the position of the test cups at the beginning of the test and maintain the chambers in this configuration throughout the test.

8. At a minimum, the following measurements should be made in at least one replicate in the control and the high and low test concentrations at the beginning of the test: temperature, dissolved oxygen, pH, and salinity.

C. Test Conditions

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE *Mysidopsis bahia* SURVIVAL, GROWTH,
AND FECUNDITY TEST**

ERL-N SOP 1.03.005
REVISION 0
JANUARY 1991
PAGE 4 OF 13

1. The light quality and intensity under ambient laboratory conditions are generally adequate. Light intensity of 10-20 $\mu\text{E}/\text{m}^2/\text{s}$, or 50 to 100 foot candles (ft.c), with a 16 h light and 8 h dark cycle and a 30 min phase-in/out period is recommended.
2. It is critical that the test water temperature be maintained at 26 - 27°C. It is recommended that the test water temperature be continuously recorded. If a water bath is used to maintain the test temperature, the water depth surrounding the test cups should be at least 2.5 cm deep.
3. The test salinity should be in the range of 20 ‰ to 30 ‰ and should not vary by more than ± 2 ‰ among the test cups on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.
4. Dissolved oxygen in test cups should not fall below 60% of saturation. The higher concentrations of some effluents will require aeration to maintain adequate DO concentrations. If one solution is aerated then all the treatments and the controls must also be gently aerated.
5. During the test, the mysids in each test cup should be fed *Artemia* nauplii less than 24-h old at the rate of 150 nauplii per mysid per day. Adding the entire daily ration at a single feeding immediately after test solution renewal may result in a significant DO depression. Therefore, it is preferable to feed half of the daily ration immediately after test solution renewal, and the second half 8 - 12 h later. Increase the feeding if the nauplii are consumed in less than 4 h. It is important that the nauplii be washed with clean seawater before introduction to the test vessels.

D. Daily Monitoring of the Test

1. The number of live mysids in each cup is counted and recorded each day before the test solutions are renewed. Dead animals and excess food should be removed with a pipette before the new test solutions are added.
2. DO, pH, temperature, and salinity should be measured in two replicates in the control and each test concentration before renewing the test medium each day. Temperature should be monitored in test cups while they are still in the water bath.
3. Remove all of the replicate cups of a concentration from the water bath and place them on a light table. Slowly pour off as much as possible of the old test medium into a 20 cm diameter culture dish on a light table. Be very careful not to strand the mysids in an empty cup or to pour out the mysids. If a mysid is inadvertently poured out of a test cup, carefully replace the mysid and note this on the datasheet.
4. Add 150 mL of new test solution slowly to each cup and replace in the water bath.

E. Termination of the Test

1. After measuring the DO, pH, temperature, and salinity and recording survival,

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE *Mysidopsis bahia* SURVIVAL, GROWTH,
AND FECUNDITY TEST**

ERL-N SOP 1.03.005
REVISION 0
JANUARY 1991
PAGE 5 OF 13

terminate the test by pouring off the test solution in all the cups to a one-cm depth.

2. The live animals must be examined for eggs and the sexes determined within 12 h of the termination of the test as follows:

- a. Examine the individual animals from each replicate under a stereomicroscope (240X) to determine the number of immature animals, the sex of the mature animals, and the presence or absence of eggs in the ovary or brood sac of the females (see Figures 2-5). This must be done while the mysids are alive because they turn opaque upon dying. This step should not be attempted by a person who has not had specialized training in the determination of sex and presence of eggs in the ovary.
- b. Record the number of immatures, males, females with eggs and females without eggs on data sheets.
- c. Pipette the mysids onto a 500 um mesh screen over a beaker and rinse with deionized water.
- d. Using forceps, place the mysids from each replicate cup on a tared weighing boat. Pieces of aluminum foil (1 cm square) or small aluminum weighing boats can be used for dry weight analyses. The weighing pans or boats should not exceed 10 mg in weight. Number each pan with a waterproof pen with the treatment concentration and replicate number. Forty-eight (48) weighing pans are required per test if organisms survive in all replicates.
- e. Dry the mysids 60°C for 24 hours.
- f. Remove the pans from the oven and transfer immediately to a desiccator. After cooling for 1 h, weigh to the nearest microgram.
- g. The minimum requirements for an acceptable test are 80% survival and an average weight of at least 0.20 mg/mysid in the controls. If fecundity in the controls is adequate (egg production by 50% of females), fecundity should be used as a criterion of effect in addition to survival and growth.

IV. TROUBLE SHOOTING

1. Toxic substances may be introduced by contaminants on glassware and testing equipment. Be sure that all equipment is thoroughly cleaned and/or soaked in clean seawater before use in a test.
2. Excess *Artemia* nauplii in exposure cups during the test may sequester metals and other toxic substances, and lower the DO. Care should be taken to avoid overfeeding mysids during the test.
3. Rooms or incubators with high volume ventilation should be used with caution

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE *Mysidopsis bahia* SURVIVAL, GROWTH,
AND FECUNDITY TEST**

ERL-N SOP 1.03.005
REVISION 0
JANUARY 1991
PAGE 6 OF 13

because the volatilization of the test solutions and evaporation of dilution water may cause wide fluctuations in salinity. Covering the test cups with clear polyethylene plastic may help prevent volatilization and evaporation of the test solutions.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data.
2. The end points of the mysid 7-day rapid-chronic test are based on the adverse effects on survival, growth, and egg development. Least effect and no effect concentrations (LOECs and NOECs), for survival, growth, and reproduction are obtained using one-way analysis of variance (ANOVA) followed by Dunnett's Procedure for comparing treatments to a control (Dunnett, 1955).
3. Data are used, with data from other toxicity tests, in assessing the toxicity of effluents and/or receiving waters. Data may also be used in the NPDES permit process.

VI. REFERENCES

- Dunnett, C.W. 1955. A multiple comparisons procedure for comparing several treatments with a control. JASA 50:1096-1101.
- US EPA. 1988. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. EPA Office of Research and Development EPA-600/4-87/028 (May 1988).

TEST: _____

START DATE: _____

SALINITY: _____

TREATMENT/ REPLICATE	DAY 1 # ALIVE	DAY 2 # ALIVE	DAY 3 # ALIVE	DAY 4 # ALIVE	DAY 5 # ALIVE	DAY 6 # ALIVE	DAY 7 # ALIVE	FEMALES W/EGGS	FEMALES NO EGGS	MALES	IMMATURES
1											
2											
3											
4											
5											
6											
7											
8											
1											
2											
3											
4											
5											
6											
7											
8											
1											
2											
3											
4											
5											
6											
7											
8											

Figure 1. Sample data sheet.

TEST: _____

START DATE: _____

SALINITY: _____

	TRTMT	TEMP	SALINITY	OO	PH	TRTMT	TEMP	SALINITY	OO	PH	
DAY 1	REP										
	REP										
DAY 2	REP										
	REP										
DAY 3	REP										
	REP										
DAY 4	REP										
	REP										
DAY 5	REP										
	REP										
DAY 6	REP										
	REP										
DAY 7	REP										
	REP										
	TRTMT	TEMP	SALINITY	OO	PH	TRTMT	TEMP	SALINITY	OO	PH	
DAY 1	REP										
	REP										
DAY 2	REP										
	REP										
DAY 3	REP										
	REP										
DAY 4	REP										
	REP										
DAY 5	REP										
	REP										
DAY 6	REP										
	REP										
DAY 7	REP										
	REP										

Fig. 1, continued.

TEST: _____

START DATE: _____

SALINITY: _____

TREATMENT REPLICATE	PAN #	TARE WT.	TOTAL WT.	ANIMAL WT.	# OF ANIMALS	\bar{X} WT./ ANIMAL
1						
2						
3						
4						
5						
6						
7						
8						
1						
2						
3						
4						
5						
6						
7						
8						
1						
2						
3						
4						
5						
6						
7						
8						

MATURE FEMALE, EGGS IN OVIDUCTS

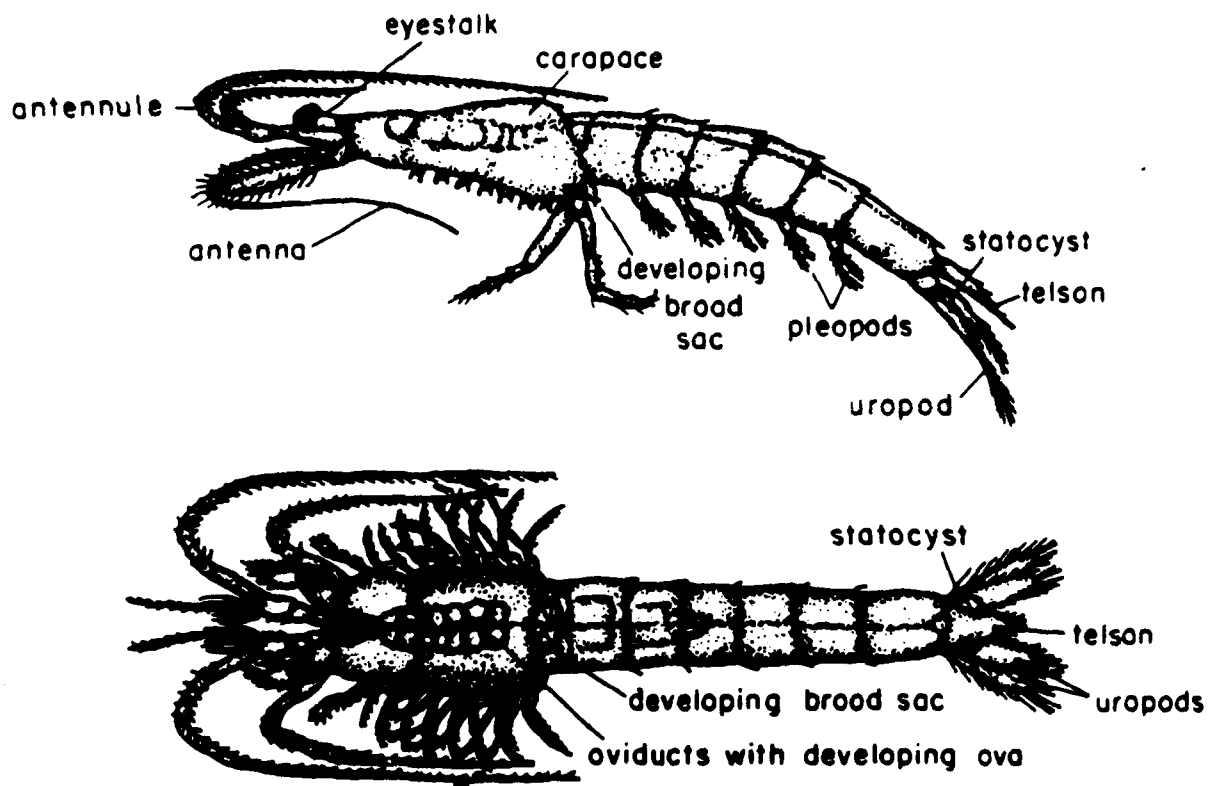


Figure 2. Mature female *M. bahia* with eggs in oviducts.
From Lussier, Kuhn, and Sewall, 1987.

MATURE FEMALE, EGGS IN BROOD SAC

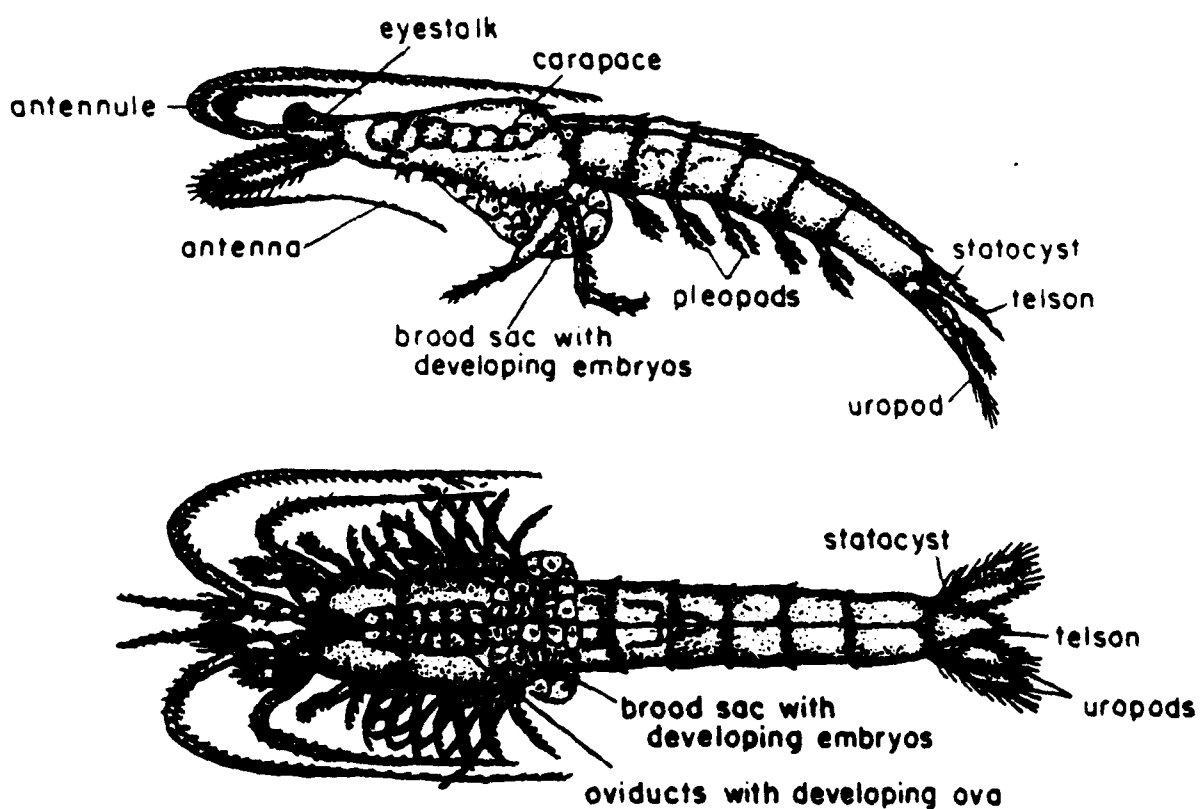


Figure 3. Mature female *M. bahia* with eggs in oviducts and developing embryos in the brood sac. Above: lateral view. Below: dorsal view. From Lussier, Kuhn, and Sewall, 1987.

MATURE MALE

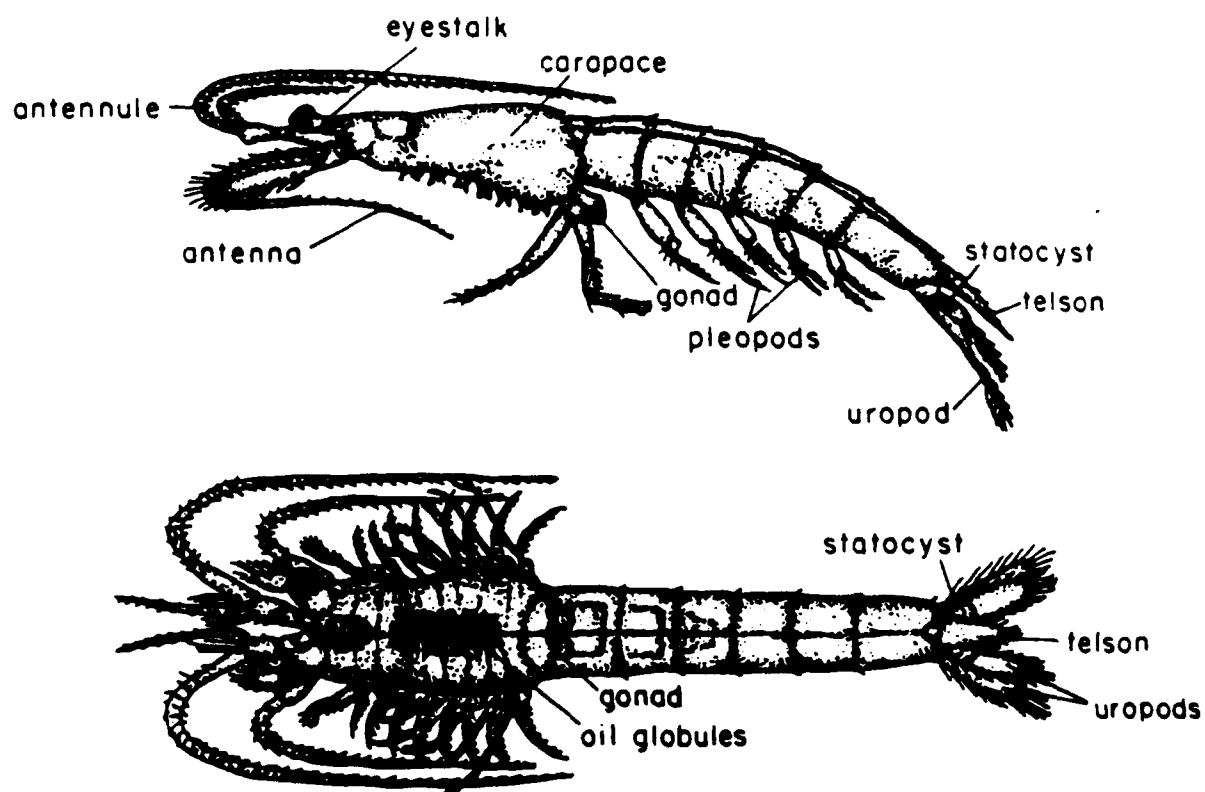


Figure 4. Mature male *M. bahia*. From Lussier, Kuhn, and Sewall, 1987.

IMMATURE

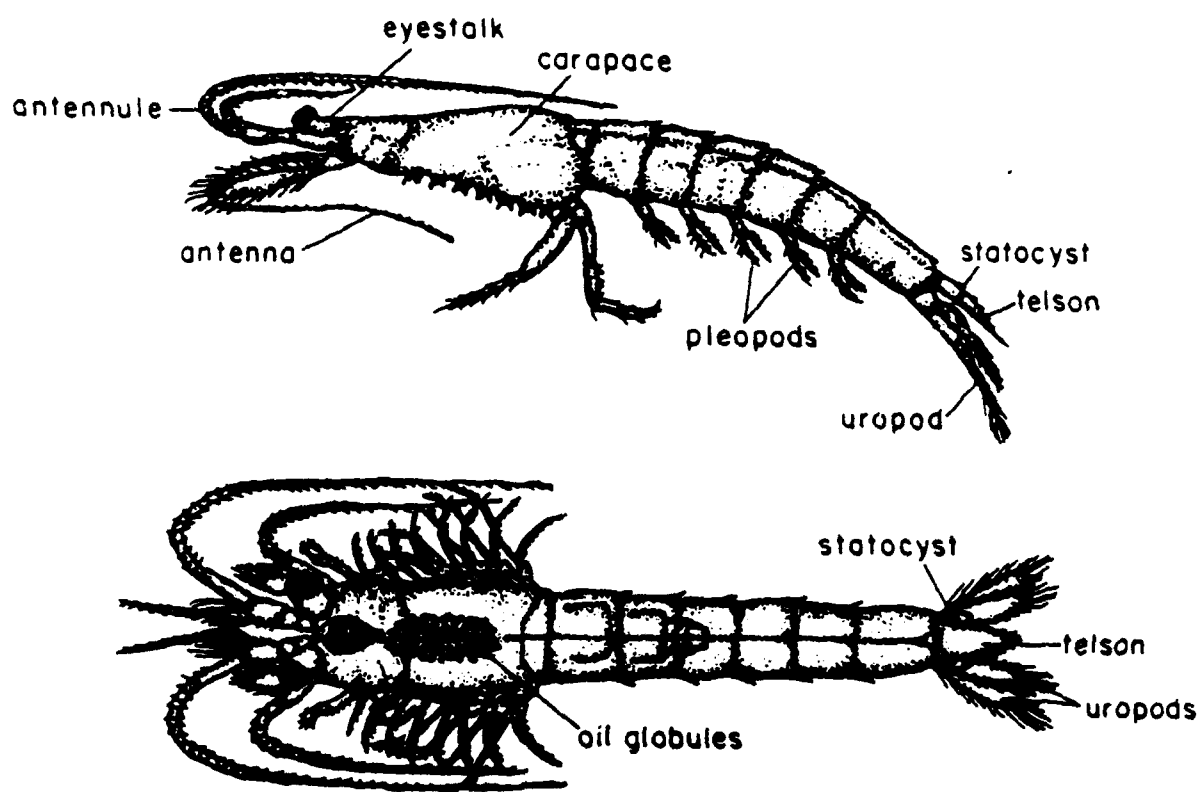


Figure 5. Immature *M. bahia*, (A) lateral view, (B) dorsal view.
 From Lussier, Kuhn, and Sewall, 1987.

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN, *Arbacia punctulata*,
FERTILIZATION TEST**

ERL-N SOP 1.03.006
REVISION 0
JANUARY 1991
PAGE 1 OF 10

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I. OBJECTIVE

This method measures the toxicity of effluent and receiving waters to the gametes of the sea urchin, *Arbacia punctulata*, during a 1 h and 20 min exposure. The purpose of the sperm cell toxicity test is to determine the concentration of a test substance that reduces fertilization of exposed gametes relative to that of the control.

II. NECESSARY MATERIALS AND EQUIPMENT

- Facilities for holding and acclimating test organisms.
- Laboratory sea urchin culture unit -- See SOP on Culture. To test effluent or receiving water toxicity, sufficient eggs and sperm must be available.
- Environmental chamber or equivalent facility with temperature control ($20 \pm 1^\circ\text{C}$) for controlling temperature during exposure.
- Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.
- Balance -- Analytical, capable of accurately weighing to 0.0001 g.
- Reference weights, Class S -- for checking performance of balance.
- Air pump -- for supplying air.
- Air lines, and air stones -- for aerating water containing adults.
- Vacuum suction device -- for washing eggs.
- pH and DO meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of these two parameters, portable, field-grade instruments are acceptable.
- Transformer, 10-12 Volt, with steel electrodes -- for stimulating release of eggs and sperm.
- Centrifuge, bench-top, slant-head, variable speed -- for washing eggs.
- Fume hood -- to protect the analyst from formaldehyde fumes.
- Dissecting microscope -- for counting diluted egg stock.
- Compound microscope -- for examining and counting sperm cells and fertilized eggs.
- Sedgwick-Rafter counting chamber -- for counting egg stock.

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN, *Arbacia punctulata*,
FERTILIZATION TEST**

ERL-N SOP 1.03.006
REVISION 0
JANUARY 1991
PAGE 2 OF 10

- Hemacytometer, Neubauer -- for counting sperm.
- Count register, 2-place -- for recording sperm and egg counts.
- Refractometer -- for determining salinity.
- Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.
- Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.
- Ice bucket, covered -- for maintaining live sperm.
- Centrifuge tubes, conical, 15 mL -- for washing eggs.
- Cylindrical glass vessel, 8-cm diameter -- for maintaining dispersed egg suspension.
- Beakers -- six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.
- Glass dishes, flat bottomed, 20-cm diameter -- to hold adults during gamete collection.
- Wash bottles -- for deionized water, for rinsing small glassware and instrument electrodes and probes.
- Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.
- Syringes, 1-mL, and 10-mL, with 18 gauge, blunt-tipped needles (tips cut off) -- for collecting sperm and eggs.
- Pipets, volumetric -- Class A, 1-100 mL.
- Pipets, automatic -- adjustable, 1-100 mL.
- Pipets, serological -- 1-10 mL, graduated.
- Pipet bulbs and fillers -- PROPIPET[®], or equivalent.
- Tape, colored -- for labelling tubes.
- Markers, water-proof -- for marking containers, etc.
- Sea Urchins (approximately 12 of each sex).
- Scintillation vials, 20 mL, disposable -- to prepare test concentrations.
- Parafilm -- to cover tubes and vessels containing test materials.
- Gloves, lab coat, disposable -- for personal protection from contamination.
- Safety glasses
- Data sheets (one set per test) -- for data recording (Figure 1).
- Acetic acid, 10%, reagent grade, in sea water -- for preparing killed sperm dilutions.
- Formalin, 10% in seawater -- for preserving eggs.
- pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check.
- Membranes and filling solutions for dissolved oxygen probe or reagents for modified Winkler analysis.
- Laboratory quality assurance samples and standards for the above methods.
- Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.
- Effluent, surface water, and dilution water.
- Saline test and dilution water -- The salinity of the test water must be 30 ‰. The salinity should vary by no more than ± 2 ‰ among the replicates.

III. METHODS

A. Test Solutions

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN, *Arbacia punctulata*,
FERTILIZATION TEST**

ERL-N SOP 1.03.006
REVISION 0
JANUARY 1991
PAGE 3 OF 10

1. Samples are used directly as collected when sample salinity is between 28 and 32 ppt. If samples do not require salinity adjustment, natural seawater is used in all washing and diluting steps and as control water. Local water from an uncontaminated area may be used as an additional control.
2. If salinity adjustment is required, prepare 3 L of control water at 30 ‰ using hypersaline brine (see SOP on Preparation of Brine). This water is used in all washing and diluting steps and as control water in the test. Natural sea water and uncontaminated local waters may be used as additional controls.
3. Effluent/receiving water samples are adjusted to salinity of 30 ‰ using hypersaline brine as necessary.
4. The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is used with this procedure, starting with a high concentration of 70% effluent (for freshwater effluents). If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used.
5. Three replicates are prepared for each test concentration, using 5 mL of solution in disposable liquid scintillation vials. A 50% (0.5) concentration series can be prepared by serially diluting test concentrations with control water.
6. All test samples are equilibrated at $20 \pm 1^{\circ}\text{C}$ before addition of sperm.

B. Collection and Preparation of Gametes for the Test

1. Select four females and place in shallow bowls, barely covering the shell with seawater. Stimulate the release of eggs by touching the test with electrodes from the transformer. Collect about 3 mL of eggs from each female using a 10 cc syringe with a blunted needle. Remove the needle from the syringe before adding the eggs to a 15 mL conical centrifuge tube. Pool the eggs. The egg stock may be held at room temperature for several hours before use. Note: The egg suspension may be prepared during the 1-h sperm exposure.
2. Select four males and place in shallow bowls, barely covering the animals with seawater. Stimulate the release of sperm by touching the shell with steel electrodes connected to a 10 - 12 V transformer (about 30 seconds each time). Collect the sperm (about 0.25 mL) from each male, using a 1 mL disposable syringe fitted with an 18-gauge, blunt-tipped needle. Maintain the syringe containing pooled sperm sample on ice. The sperm must be used in a toxicity test within 1 h of collection.
3. Using control water, dilute the pooled sperm sample to a concentration of about 5×10^7 sperm/mL (SPM). Estimate the sperm concentration as described below:
 - a. Make a sperm dilutions of 1:50, 1:100, 1:200, and 1:400, using 30‰ seawater, as follows:

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN, *Arbacia punctulata*,
FERTILIZATION TEST**

ERL-N SOP 1.03.006
REVISION 0
JANUARY 1991
PAGE 4 OF 10

1. Add 400 μ L of collected sperm to 20 mL of sea water in Vial A. Cap Vial A and mix by inversion.
 2. Add 10 mL of sperm suspension from Vial A to 10 mL of seawater in Vial B. Cap Vial B and mix by inversion.
 3. Add 10 mL of sperm suspension from Vial B to 10 mL of seawater in Vial C. Cap Vial C and mix by inversion.
 4. Add 10 mL of sperm suspension from Vial C to 10 mL of seawater in Vial D. Cap Vial D and mix by inversion.
 5. Discard 10 mL from Vial D. (The volume of all suspensions is 10 mL).
- b. Make a 1:2000 killed sperm suspension and determine the SPM.
1. Add 10 mL 10% acetic acid in seawater to Vial C. Cap Vial C and mix by inversion.
 2. Add 1 mL of killed sperm from Vial C to 4 mL of seawater in Vial E. Mix by gentle pipetting with a 4-mL pipetter.
 3. Add sperm from Vial E to both sides of the Neubauer hemacytometer. Let the sperm settle 15 min.
 4. Count the number of sperm in the central 400 squares on both sides of the hemacytometer using a compound microscope (400X). Average the counts from the two sides.
 5. SPM in Vial E = $10^4 \times$ average count.
- c. Calculate the SPM in all other suspensions using the SPM in Vial E above:
- $$\begin{aligned}\text{SPM in Vial A} &= 40 \times \text{SPM in Vial E} \\ \text{SPM in Vial B} &= 20 \times \text{SPM in Vial E} \\ \text{SPM in Vial D} &= 5 \times \text{SPM in Vial E} \\ \text{SPM in original sperm sample} &= 2000 \times \text{SPM in Vial E}\end{aligned}$$
- d. Dilute the sperm suspension with a sperm concentration greater than 5×10^7 SPM to 5×10^7 SPM.
- $$\text{Actual SPM} / (5 \times 10^7) = \text{dilution factor (DF)}$$
- $$[(\text{DF}) \times 10] - 10 = \text{mL of seawater to add to vial.}$$
- e. Confirm the sperm count by sampling from the test stock. Add 0.1 mL

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN, *Arbacia punctulata*,
FERTILIZATION TEST**

ERL-N SOP 1.03.006
REVISION 0
JANUARY 1991
PAGE 5 OF 10

of test stock to 9.9 mL of 10% acetic acid in seawater, and count with the hemacytometer. The count should average 50 ± 5 .

4. Wash the pooled eggs three times using control water with gentle centrifugation (500xg or lowest possible setting) for 3 min using a tabletop centrifuge. If the wash water becomes red, the eggs have lysed and must be discarded.

a. Dilute the egg stock, using control water, to 2000 ± 200 eggs/mL.

1. Remove the final wash water and transfer (by filling the centrifuge tube with control water and repeatedly inverting to resuspend the eggs) the washed eggs to a beaker containing a small volume (about 50 mL) of control water. Add more control water to bring the eggs to a volume of 200 mL ("egg stock").

2. Mix the egg stock using gentle aeration. Cut the point from a pipet tip, then transfer 1 mL of eggs from the egg stock to a vial containing 9 mL of control water. (This vial contains an egg suspension diluted 1:10 from egg stock).

3. Mix the contents of the vial using gentle pipetting. Cut the point from a pipet tip, then transfer 1 mL of eggs from the vial to a Sedgwick-Rafter counting chamber. Count all eggs in the chamber using a dissecting microscope ("egg count").

4. Calculate the concentration of eggs in the stock. $\text{Eggs/mL} = 10 \times (\text{egg count})$. Dilute the egg stock to 2000 eggs/mL by the formula below.

b. If the egg count is equal to or greater than 200:

$$(\text{egg count}) - 200 = \text{volume (mL) of control water to add to egg stock}$$

c. If the egg count is less than 200, allow the eggs to settle and remove enough control water to concentrate the eggs to greater than 200, repeat the count, and dilute the egg stock as above.

NOTE: It requires 18 mL of a egg stock solution for each test with a control and five exposure concentrations (three replicates).

d. Transfer 1 mL of the diluted egg stock to a vial containing 9 mL of control water. Mix well, then transfer 1 mL from the vial to a Sedgwick-Rafter counting chamber. Count all eggs using a dissecting microscope. Confirm that the final egg count = 200 ± 20 per mL.

B. Start of the Test

1. Within 1 h of collection add 100 μL of appropriately diluted sperm to each test vial. Record the time of sperm addition.

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN, *Arbacia punctulata*,
FERTILIZATION TEST**

ERL-N SOP 1.03.006
REVISION 0
JANUARY 1991
PAGE 6 OF 10

2. Incubate all test vials at $20 \pm 1^{\circ}\text{C}$ for 1 h.
3. Mix the diluted egg suspension (2000 eggs/mL), using gentle bubbling. Add 1 mL of diluted egg suspension to each test vial using a wide mouth pipet tip. Incubate 20 min at $20 \pm 1^{\circ}\text{C}$.

C. Termination of the Test

1. Terminate the test and preserve the samples by adding 2 mL of 10% formalin in seawater to each vial.
2. Vials may be evaluated immediately or capped and stored for as long as one week before being evaluated.
3. To determine fertilization, transfer about 1 mL eggs from the bottom of a test vial to a Sedgwick-Rafter counting chamber. Observe the eggs using a compound microscope (100 X). Count about 100 eggs/sample. Record the number counted and the number unfertilized. Fertilization is indicated by the presence of a fertilization membrane surrounding the egg. Adjustment of the microscope to obtain proper contrast may be required to observe the fertilization membrane.
NOTE: Because samples are fixed in formalin, a ventilation hood is set-up surrounding the microscope to protect the analyst from prolonged exposure to formaldehyde fumes.

D. Acceptability of Test Results

1. The sperm:egg ratio routinely employed should result in fertilization of at least 50% of the eggs in the control chambers.

IV. TROUBLE SHOOTING

1. Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data. Calculate the percent of unfertilized eggs for each replicate.
2. The endpoints of toxicity tests using the sea urchin are based on the reduction in percent of eggs fertilized. An estimate of the effluent concentration which would cause a 50% reduction in egg fertilization (EC50) is calculated using Trimmed Spearman-Kärber analysis (Hamilton, Russo, and Thurston, 1977). Dunnett's Procedure (Dunnett, 1955) is used to estimate no effect and least effect concentrations (NOEC and LOEC values).

3. Data are used along with other toxicity tests in assessing the toxicity of an effluent or receiving water.

VI. REFERENCES

- Dunnett, C.W. 1955. A multiple comparisons procedure for comparing several treatments with a control. *JASA* 50:1096-1101.
- Hamilton, M.A., R.C. Russo, and R.V. Thurston. 1977. Trimmed Spearman-Kärber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol.* 11(7):714-719.
- US EPA. 1988. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms. Weber, C.I., et al (eds). EPA Office of Research and Development EPA-600/4-87/028 (May 1988).

SPERM CELL TOXICITY TEST
DATA SHEET # 1

TESTID:
PERFORMED BY:

SPERM SOLUTIONS

HEMACYTOMETER CT, E: $\times 10^4$ = spm 'E'
sperm concentrations: E x 40 = A = SPM
E x 20 = B = SPM
E x 5 = D = SPM

SOLUTION SELECTED FOR TEST ($\geq 5 \times 10^7$ SPM):
DILUTION: $\text{SPM} / (5 \times 10^7)$ = DF
((DF) x 10) - 10 = + SW, ML

EGG SOLUTIONS

INITIAL COUNT: - VOLUME FOR FINAL EGG STOCK

COUNT ON FINAL EGG STOCK: (200 x 10 EGGS/ML)

TEST STOCKS

SPERM STOCK: (5×10^7 SPM)
VOLUME ADDED/TEST VIAL: (100 UL)
EGG STOCK: (2000/ML)
VOLUME ADDED/TEST VIAL (1 ML)

TEST TIMES

SPERM COLLECTION:

SPERM ADDED:

EGGS ADDED:

FIXITIVE ADDED:

SAMPLES READ:

SALINITIES:

VERIFICATION:

TESTID:

STUDY:

PROJECT:

SCONTROL1:

SCONTROL2:

SCONTROL3:

EFF1:

EFFCTL1:

EFF2:

EFFCTL2:

EFF3:

EFFCTL3:

TESTCOM:

SAMPLE COMMENT:						
SAMPNUM	SAMPTYPE	TREAT	RCONC	REP	CTD	UNFERT
				1	100	
				2	100	
				3	100	
General Comments:						

SAMPLE COMMENT:						
SAMPNUM	SAMPTYPE	TREAT	RCONC	REP	CTD	UNFERT
				1	100	
				2	100	
				General Comments:		

SAMPLE COMMENT:						
SAMPNUM	SAMPTYPE	TREAT	RCONC	REP	CTD	UNFERT
				1	100	
				2	100	
				3	100	
General Comments:						

SAMPLE COMMENT:						
SAMPNUM	SAMPTYPE	TREAT	RCONC	REP	CTD	UNFERT
				1	100	
				2	100	
				3	100	
General Comments:						

SPERM CELL TEST DATA ANALYSES RECORD

TESTID: _____

TEST COMMENT: _____

VERIFICATION: _____

	<u>DATE</u>	<u>INITIALS</u>
TEST EVALUATED:	_____	_____
DATA ENTERED INTO RAW SCT DATABASE (@ADDDRAW OR @NEWSCT):	_____	_____
DATA CORRECT:	_____	_____
DATA SUBSET (@PROCSCT):	_____	_____
SUBSET DATA CORRECT:	_____	_____
SAS SENT (@SPERMAOV):	_____	_____
SAS RETURNED/CHECKED, DIFFERENCES NOTED, NOECS DETERMINED:	_____	_____
SPEARMAN EC50'S CALCULATED:	_____	_____
DIFFERENCES (*), EC50'S AND NOECS ADDED INTO TESTDB DATABASE: (@CHANGESTEST)	_____	_____
RESULTS DROPPED: (@ADDSPRM)	_____	_____
DATA AND RESULTS QA'D/CORRECTED:	_____	_____
DATA AND RESULTS COPIED, SIGNED, FILED:	_____	_____
RESULTS DISTRIBUTED:	_____	_____

ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN LARVAL
DEVELOPMENT TEST

ERL-N SOP 1.03.007
REVISION 0
DECEMBER 1990
PAGE 1 OF 15

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I. OBJECTIVE

The purpose of the sea urchin larval development test is to determine the effects of effluents and water samples on survival, growth, and development of larvae of the sea urchin, *Arbacia punctulata*.

II. NECESSARY MATERIALS AND EQUIPMENT

- Facilities for holding and acclimating test organisms.
- Laboratory sea urchin culture unit -- See culturing SOP. To test effluent or receiving water toxicity, sufficient eggs and sperm must be available.
- Environmental chamber or equivalent facility with temperature control ($20 \pm 1^\circ\text{C}$) for controlling temperature during exposure.
- Water purification system -- Millipore Super-Q, Deionized water (DI) or equivalent.
- Balance -- Analytical, capable of accurately weighing to 0.0001 g.
- Reference weights, Class S -- for checking performance of balance.
- Air pump -- for supplying air.
- Air lines, and air stones -- for aerating water containing adults.
- Vacuum suction device -- for washing eggs.
- pH and DO meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of these two parameters, portable, field-grade instruments are acceptable.
- Transformer, 10-12 Volt, with steel electrodes -- for stimulating release of eggs and sperm.
- Centrifuge, bench-top, slant-head, variable speed -- for washing eggs.
- Fume hood -- to protect the analyst from formaldehyde fumes.
- Dissecting microscope -- for counting diluted egg stock.
- Compound microscope -- for examining and counting sperm cells and fertilized eggs.
- Compound microscope with CCD digital camera and low powered objectives (2-

ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN LARVAL
DEVELOPMENT TEST

ERL-N SOP 1.03.007
REVISION 0
DECEMBER 1990
PAGE 2 OF 15

- 10x magnification) -- for use with image analyzer (quantification of growth endpoint).
- Cambridge Instruments Quantimet 520 image analyzer with IBM PC/AT (or equivalent) and video display -- for quantification of growth endpoint.
 - Sedgwick-Rafter counting chamber -- for counting egg stock and final examination of larvae.
 - Hemacytometer, Neubauer -- for counting sperm.
 - Count register, 2-place -- for recording sperm and egg counts.
 - Refractometer -- for determining salinity.
 - Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.
 - Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.
 - Ice bucket, covered -- for maintaining live sperm.
 - Centrifuge tubes, conical, 15 mL -- for washing eggs.
 - Cylindrical glass vessel, 8-cm diameter -- for maintaining dispersed egg suspension.
 - Beakers -- at least six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.
 - Glass dishes, flat bottomed, 20-cm diameter -- for holding adult urchins during gamete collection.
 - Wash bottles -- for deionized water, for rinsing small glassware and instrument electrodes and probes.
 - Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.
 - Syringes, 1-mL, and 10-mL, with 18 gauge, blunt-tipped needles (tips cut off) -- for collecting sperm and eggs.
 - Pipets, volumetric -- Class A, 1-100 mL.
 - Pipets, automatic -- adjustable, 1-100 mL.
 - Pipets, serological -- 1-10 mL, graduated.
 - Pipet bulbs and fillers -- PROPIPET[®], or equivalent.
 - Tape, colored -- for labelling tubes.
 - Markers, water-proof -- for marking containers, etc.
 - Sea Urchins (approximately 12 of each sex).
 - Scintillation vials, 20 mL, disposable -- to prepare test concentrations.
 - Parafilm -- to cover tubes and vessels containing test materials.
 - Gloves, lab coat, disposable -- for personal protection from contamination.
 - Safety glasses.
 - Data sheets (one set per test) -- for data recording (Figure 1).
 - Acetic acid, 10%, reagent grade, in sea water -- for preparing killed sperm dilutions.
 - Formalin, 10% in seawater -- for preserving eggs.
 - pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check.
 - Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.
 - Effluent, surface water, and dilution water.
 - Saline test and dilution water -- The salinity of the test water must be 30 ‰. The salinity should vary by no more than ± 2 ‰ among the replicates.

III. METHODS

A. Test Solutions

1. Samples are used directly as collected when sample salinity is between 28 and 32 parts per thousand. If samples do not require salinity adjustment natural seawater is used in all washing and diluting steps. Local uncontaminated water may be used as an additional control.
2. If salinity adjustment is required, prepare 3 L of control water at 30 ‰ using hypersaline brine (see Brine SOP). This water is used in all washing and diluting steps and as control water in the test. Natural sea water and uncontaminated local waters may be used as additional controls.
3. Effluent/receiving water samples are adjusted to salinity of 30 ‰.
4. The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is used with this procedure, starting with a high concentration of 70% effluent (for freshwater effluents). If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used.
5. Three replicates are prepared for each test concentration, using 10 mL of solution in disposable liquid scintillation vials. A 50% (0.5) concentration series can be prepared by serially diluting test concentrations with control water.
6. All test samples are equilibrated at $20 \pm 1^{\circ}\text{C}$ before addition of sperm.

B. Collection and Preparation of Gametes for the Test

1. Select four females and place in shallow bowls, barely covering the shell with seawater. Stimulate the release of eggs by touching the test with electrodes from the transformer. Collect about 3 mL of eggs from each female using a syringe with a blunted needle. Remove the needle from the syringe before adding the eggs to a 15 mL conical centrifuge tube. Pool the eggs. The egg stock may be held at room temperature for several hours before use. Note: The egg suspension may be prepared during the 1-h sperm exposure.
2. Select four males and place in shallow bowls, barely covering the animals with seawater. Stimulate the release of sperm by touching the shell with steel electrodes connected to a 12 V transformer (about 30 seconds each time). Collect the sperm (about 0.25 mL) from each male, using a 1 mL disposable syringe fitted with an 18-gauge, blunt-tipped needle. Maintain the syringe containing pooled sperm sample on ice. The sperm must be used in a toxicity test within 1 h of collection.
3. Using control water, dilute the pooled sperm sample to a concentration of about

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN LARVAL
DEVELOPMENT TEST**

ERL-N SOP 1.03.007
REVISION 0
DECEMBER 1990
PAGE 4 OF 15

5×10^7 sperm/mL (SPM). Estimate the sperm concentration as described below:

a. Make a sperm dilutions of 1:50, 1:100, 1:200, and 1:400, using 30‰ seawater, as follows:

1. Add 400 μ L of collected sperm to 20 mL of sea water in Vial A. Mix by gentle pipetting using a 5-mL pipetter.
2. Add 10 mL of sperm suspension from Vial A to 10 mL of seawater in Vial B. Mix by gentle pipetting using a 5-mL pipetter.
3. Add 10 mL of sperm suspension from Vial B to 10 mL of seawater in Vial C. Mix by gentle pipetting using a 5-mL pipetter.
4. Add 10 mL of sperm suspension from Vial C to 10 mL of seawater in Vial D. Mix by gentle pipetting using a 5-mL pipetter.
5. Discard 10 mL from Vial D. (The volume of all suspensions is 10 mL).

b. Make a 1:2000 killed sperm suspension and determine the SPM.

1. Add 10 mL 10% acetic acid in seawater to Vial C. Cap Vial C and mix by inversion.
2. Add 1 mL of killed sperm from Vial C to 4 mL of seawater in Vial E. Mix by gentle pipetting with a 5-mL pipetter.
3. Add sperm from Vial E to both sides of the Neubauer hemacytometer. Let the sperm settle 15 min.
4. Count the number of sperm in the central 400 squares on both sides of the hemacytometer using a compound microscope (400X). Average the counts from the two sides.
5. SPM in Vial E = 10^4 x average count.

c. Calculate the SPM in all other suspensions using the SPM in Vial E above:

SPM in Vial A = 40 x SPM in Vial E
SPM in Vial B = 20 x SPM in Vial E
SPM in Vial D = 5 x SPM in Vial E
SPM in original sperm sample = 2000 x SPM in Vial E

d. Dilute the sperm suspension with a concentration greater than 5×10^7 SPM to 5×10^7 SPM.

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN LARVAL
DEVELOPMENT TEST**

ERL-N SOP 1.03.007
REVISION 0
DECEMBER 1990
PAGE 5 OF 15

Actual SPM/(5 x 10⁷) = dilution factor (DF)

[(DF) x 10] - 10 = mL of seawater to add to vial.

4. Wash the pooled eggs three times using control water with gentle centrifugation (500xg or the lowest possible setting) for 3 min using a tabletop centrifuge). If the wash water becomes red, the eggs have lysed and must be discarded.

a. Dilute the egg stock, using control water, to about 2000 \pm 200 eggs/mL.

1. Remove the final wash water from the eggs and transfer the washed eggs (by refilling the centrifuge tube with control water and repeatedly inverting to resuspend the eggs) to a beaker containing a small amount (about 50 mL) of control water. Add control water to bring the eggs to a volume of 200 mL ("egg stock").

2. Mix the egg stock using gentle aeration. Cut the point from a pipet tip and transfer 1 mL of eggs from the egg stock to a vial containing 9 mL of control water. (This vial contains an egg suspension diluted 1:10 from egg stock).

3. Mix the contents of the vial using gentle pipetting. Cut the point from a pipet tip and transfer 1 mL of eggs from the vial to a Sedgwick-Rafter counting chamber. Count all eggs in the chamber using a dissecting microscope at 10X ("egg count").

4. Calculate the concentration of eggs in the stock. Eggs/mL = 10x (egg count). Dilute the egg stock to 2000 eggs/mL by the formula below.

b. If the egg count is equal to or greater than 200:

(egg count) - 200 = volume (mL) of control water to add
to egg stock

c. If the egg count is less than 200, allow the eggs to settle and remove enough control water to concentrate the eggs to greater than 200, repeat the count, and dilute the egg stock as above. 100 mL of egg stock are required to perform this test.

d. Transfer 1 mL of the diluted egg stock to a vial containing 9 mL of control water. Mix well, then transfer 1 mL from the vial to a Sedgwick-Rafter counting chamber. Count all eggs using a dissecting microscope. Confirm that the final egg count = 200/mL \pm 20.

5. Mix the egg stock well, subsample 100 mL, and place the subsample in a clean beaker. Add 10 mL of the proper sperm dilution to the beaker and mix well. This will result in a egg:sperm ratio of 1:2500, which should allow acceptable egg

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN LARVAL
DEVELOPMENT TEST**

ERL-N SOP 1.03.007
REVISION 0
DECEMBER 1990
PAGE 6 OF 15

fertilization 1 hr after sperm addition.

C. Start of the Test

1. Mix the diluted embryo suspension (2000 embryos/mL), using gentle aeration. Add 1 mL of diluted egg suspension to each test vial using a wide mouth pipet tip. Incubate covered for 48 hours $20 \pm 1^\circ\text{C}$.

D. Termination of the Test

1. Terminate the test and preserve the samples by adding 2 mL of 10% formalin in seawater to each vial.
2. Vials may be evaluated immediately or capped and stored for as long as one week before being evaluated.
3. Each vial is thoroughly mixed and a 1 mL aliquot added to a Sedgwick-Rafter counting chamber for microscopic observation and image analysis. The total number of larvae and of appropriately developed larvae (pluteii) are counted to determine survival and development for each treatment. Fifty larvae per replicate are also observed using the image analysis system and measured for maximum length, total area, and shape (a function relating observed shape to that of a circle).

IV. TROUBLE SHOOTING

1. Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data.
2. An estimate of the effluent concentration which would cause a 50% toxic effect (EC50) for each parameter is calculated using Trimmed Spearman-Kärber analysis (Hamilton, Russo, and Thurston, 1977). One-way analysis of variance (ANOVA) followed by Dunnett's Procedure (Dunnett, 1955) is used to compare single treatments to the control in order to estimate no effect and least effect concentrations (NOEC and LOEC values).
3. Data are used along with other toxicity tests in assessing the toxicity of an effluent or receiving water.

VI. REFERENCES

Dunnett, C.W. 1955. A multiple comparisons procedure for comparing several treatments

**ERL-N STANDARD OPERATING PROCEDURE
CONDUCTING THE SEA URCHIN LARVAL
DEVELOPMENT TEST**

**ERL-N SOP 1.03.007
REVISION 0
DECEMBER 1990
PAGE 7 OF 15**

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VII. APPENDIX A

Sea Urchin Larval Measurement Basic Users Guide

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3/16/90

This document briefly details the theory behind and the standard operating procedure for the sea urchin larval measurement system as developed at ERL/N for the Cambridge Instruments Quantimet 520 series of image analyzers. Image analysis can be subdivided into three logical operations image acquisition, enhancement and quantification. This discussion will correlate the use of the measurement system with these three basic procedures.

The system was written in QBASIC, a subset of the basic syntax, which runs under the interactive Q520 environment and provides access to most of the image analysis subroutines. The image system is initialized by turning on the Q520 unit, the PC/AT, and the video display monitors. The image software is initialized by typing: start <cr> (where <cr> denotes a carriage return.). The message: downloading xxx.xxx will appear. When data transmission is complete the user is presented with the main level of the Quantimet interactive system (figure 1).

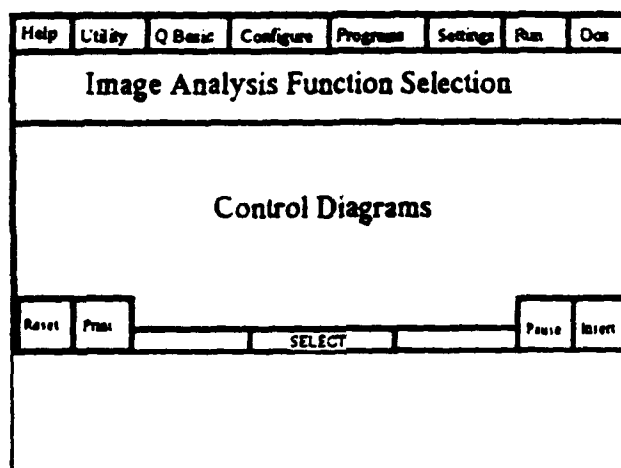


Figure 1 Q520 Interactive System

The interactive system is mouse driven, menu selections are made by positioning the mouse cursor over the desired item and pressing the center mouse button. The action of selecting and activating an option with the mouse cursor will hence forth be referred to as "clicking on" an option. To load the urchin software click on the option Q Basic at the top of the screen. The user will then be presented with the prompt "=>". To load the software type: "load baby2" <cr> "run" <cr>. The user will then be presented with the opening screen (figure 2).

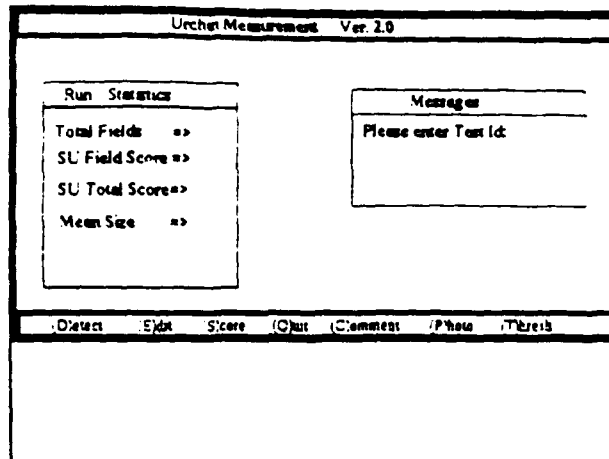


Figure 2 Opening Screen

The opening screen is divided into three logical areas:

1) Run Statistics.

This section gives important feedback on the current measurements.

Output includes:

- Total fields processed
- Sea Urchin field score
- Total Sea Urchin Field Score
- Mean Size (surface area)

2) Messages:

This section is essentially a dialogue box which provides instructions and serves a data entry area.

3) Control Panel (lower menu area)

This section consists of a number of one work commands. Commands are selected by pressing the key enclosed by parenthesis. Commands currently available are:

- (D)etect: Causes the currently masked region to be measured.
- (E)dit: Allows the user to edit the overlay plane using the digitizing tablet.
- (S)core: Terminates the session and saves all data to a file.
- (Q)uit: Terminates the session and disguards all data.

- (C)omment: Allows the user to attach a one line comment field to any data point.
- (P)hoto: Stores the currently displayed image in a data file.
- (T)hresh: Allows the user to re-threshold an image.

When the opening screen appears the user will see the following in the message box: "Please enter the slide ID: ", the user inputs a string no longer than six characters and presses <cr>. The user is next prompted for a test id and a similar reply (except that there is a 32 character limit) is made. After the initial information has been provided the user is presented with the calibration screen (Figure 3). The operator clicks on the appropriate objective size and then clicks on the "continue" box in the lower right hand corner of the screen.

Please Select objective:						
Incident	x	x	x	x	x	x
Transmitted	2.5	10.0	20.0	40.0	100.0	1
Macro Viewer	1	1	1	1	1	1

Help
Select
Continue

Figure 3
Objective selection screen.

After the proper objective has been selected the user must now obtain a good image on the display monitor. The user is presented with a screen which allows him to adjust the gain and offset of the camera (Figure 4: gain is analogous to brightness offset is analogous to contrast). After a satisfactory image has been obtained click on the continue box.

Please set gain and offset.

gain

offset

12

2

Help

Select

Continue

Figure 4

Offset and Gain

After the gain and offset have been set and continue has been selected the user is presented with the threshold screen (Figure 5). In order to make an accurate measurement the animal(s) must be entirely masked by the red overlay plane. The mask is adjusted by adjusting the threshold. As the threshold is increased (with 0 representing absolute black and 255 representing absolute white) the overlay will cover all parts of the image which contain grey scale data less than the current threshold value. It is important to obtain at least a complete outline of the animals so that the filling operator can mask the whole pludius.

When an appropriate threshold has been selected click on continue. The user may now either select (D)etect (to perform a measurement) or edit (to eliminate unwanted features). For a further explanation of the (E)dit selection see the Quantimet 520 users manual.

When the proper number of animals/fields have been processed the session may be terminated and the data stored by selecting (S)core. To begin processing another slide type run from the Qbasic prompt and repeat the previous procedure.

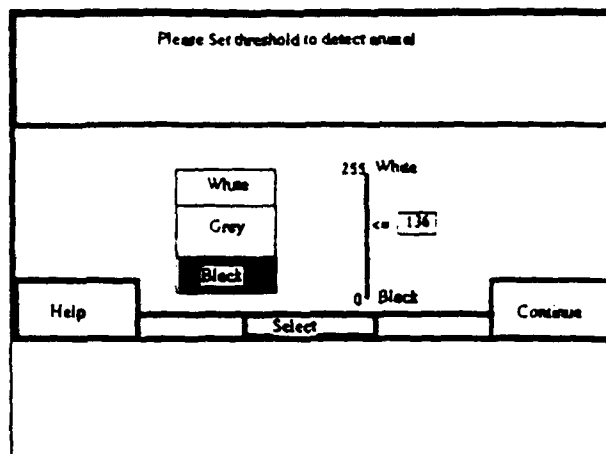


Figure 5 Threshold Detection

```

20 rem ***** Urchin screening ver 2.0 *****
30 rem ***** Manual Detection only algorithm *****
40 rem ***** Robert Petrocelli 5/21/89 *****
50 rem ***** *****
60 rem *****
61 total = 0 rem ***** animal count total
62 starttime$ = time$ rem ***** starttime
63 s = 0
64 ab = 0
66 mean = 9000 rem ***** starting mean this is redefined at runtime
67 stddev = 1000 rem ***** starting std deviation
68 stotal = 0 :loop = 0
70 disablejoy
71 enablejoy rem **** reset joystick
72 pc = 0
80 rem * variables are initialized *
90 rem
100 rem *****
110 rem Set up needed arrays *****
120 rem *****
130 rem
140 dim ff(500,6) rem ***** initial feature holder
150 dim ff2(500,4) rem **** secondary feature holder
160 dim greyarray(300) rem **** holds grey bins
170 dim num(3) rem ***** holds numff
175 dim comments$(200) rem *****holding structure for comment field
180 dim xyc(300,2) rem holds xy-cartesian cords
181 dim num2(3) rem holds feat count
182 dim num3(3)
183 dim areabins(300) rem ***** accumulation of area measures
184 dim limits(100)
190 dim xys(300,2) rem holds xy-stage cords
201 rem *** initialize frequency distribution
202 for x = 1 to 65
203 limits(x) = 200*x
204 next x
206 display 0 1 2 3
210 rem *****
220 rem paint the initial screen *****
230 rem *****
240 blankline$ = \
"
"
250 coltext 46: print chr$(27);"[2J"
260 postext 0 0: coltext 44: coltext 37: print\
" Urchin Measurement Ver 2.0 "
270 boxwidth$ = "
275 coltext 41:postext 2 1:print boxwidth$
276 coltext 30: postext 2,1:print " Run Statistics "
280 postext 1 1:coltext 47
290 for i = 1 to 10
291 postext i+2 1: print boxwidth$
292 next i
299 COLTEXT 30
300 postext 4 1:print "Total Fields ->"
310 postext 6 1:print "SU Field Score ->"
320 postext 8 1:print "SU Total Score ->"
330 postext 10 1:print "Mean Size ->"
340 rem
350 boxwidth2$ = "

```

```

370 print boxwidth2$
380 coltext 47
390 for i = 1 to 5
400 postext i+2 30:print boxwidth2$
410 next i
415 enablejoy
416 rem ***** Set up menu bar area
420 coltext 30:coltext 43:postext 2 30:print "          Messages          "
430 coltext 44:postext 23,0:print blankline$
440 postext 23,3:coltext 43:print "(D)etect"
442 postext 23 70:print "(T)hresh"
445 postext 23,16:print "(E)dit"
450 postext 23,26:print "(S)core"
460 postext 23,36:print "(Q)uit"
461 postext 23 60:print "(P)hoto"
462 postext 23 46:print "(C)omment"
463 coltext 47:coltext 30:postext 3 30:input "Please enter test id " tid$
465 coltext 47:coltext 30:postext 3 30:input "please enter slide id " id$
467 coltext 47:postext 3 30:print boxwidth2$
470 coltext 47:postext 3 30:print boxwidth2$
471 pausetext 1,"please select objective"
472 qmenu "calibrate"
473 fac = calvalue
474 fac2 = fac*fac
478 pausetext 1,"please adjust image offset and gain"
479 qmenu "image setup"
480 pausetext 1,"Please adjust threshold to detect the Animal"
481 qmenu "detect"
482 riasettings "det black",lob
483 setdist 1,limits(1)
484 pausetext 1,"Edit touching features"
485 clrfeatpar:flag =0:spec = 0
486 clraccept
490 selfeatpar 1:selfeatpar 8:selfeatpar 12:selfeatpar 15:selfeatpar 63
499 low = mean - 3*stddev:hi = mean + 3*stddev
500 nuclevel = lob:nuctop = 255
501 enablejoy
509 rem *** start loop to detect a keypress *****
510 a$ =inkey$:if a$ = "" then goto 510
520 if a$ = "q" then goto 2680
530 if a$ = "s" then goto 685
540 if a$ = "d" then goto 556
545 if a$ = "c" then goto 2700
546 if a$ = "t" then goto 480
550 if a$ = "e" then flag = 1
555 if a$ = "P" then spec = 1:pc = pc+1:postext 3 30:print "flag on":goto 510
556 if spec =1 then place$ = str$(pc)
557 if spec = 1 then greystoreon:grabon 1
558 if spec =1 postext 3 30:print "Photo Being Stored!"
559 if spec =1 saveim "c:\bobp\urchin\"+id$+" "+place$
560 if spec =1 coltext 47:postext 3 30:print boxwidth2$ :greystoreoff
561 detect nuclevel,nuctop,4,0,1,0
562 if flag =1 then qmenu "edit"
563 s = 0
564 amend "fill" 1 1
565 measfeat 1,0,0,0
570 acceptfeat 1,0.25*mean,4.0*mean
576 acceptfeat 15 1000 30000
580 rfeatnum num(1)

```

```

582 rem
583 rem ***** output to runtime statistics box
590 coltext 47:coltext 30: posttext 6 18:print s
651 distfeat 1,1,1
652 rdist 1 areabins(1)
654 posttext 10 18:print int(fac2* areabins(67))
655 mean = areabins(67)
656 rem stddev = sqrt(areabins(68))
657 if mean < 5000 then mean = 9000
660 rem
674 if s = 0 then goto 680
675 getfeates(ff2(1,1))
676 for i = 1 to s
677 ff(total+i,1) = ff2(i,1):ff(total+i,2) = ff2(i,2):\
ff(total+i,3)=ff2(i,3):ff(total+i,4) = ff2(i,4):ff(total+i,5) = spec:\
ff(total+i,6) = loop+1
678 next i
679 total = total + s:posttext 8 18:print total
680 clearplane -1
681 loop = loop+ 1: posttext 4 18:print loop
682 goto 485
685 rem
688 coltext 47
695 endtimes$ = times$
696 rem ***** output to data file designated by slide id
700 open #1 "c:\bobb\urchin\"+id$+".urc"
710 print #1: "Test Slide Animal Animal total a 1 "\
"w s flag "
720 print #1: blankline$
900 for i = 1 to total
2110 print #1: tid$ id$ " " " i " "total\
" "ff(i,1)*(fac2) " "ff(i,2)*fac " " \
" "ff(i,3)*fac" "ff(i,4)/1000" "ff(i,5)" "comments$(ff(i,6))
2120 print #1: blank$
130 next i
2200 close #1
2680 cls
2690 print "program terminated normally" :goto 2900
2700 posttext 3 30 :print "Enter a Comment line:"
2710 posttext 4 30 :input comments$(loop+1)
2720 coltext 47:posttext 3 30:print boxwidth2$
2730 posttext 4 30:print boxwidth2$
2740 a$ = "":goto 510
2900 end
$

```

**ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST**

ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 1 OF 18

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I. OBJECTIVE

This method was prepared as an appendix to the marine chronic toxicity test manual (EPA/600/4-87/028) for use in the National Pollutant Discharge Elimination System (NPDES). This test method uses the embryos of the euryhaline clam, *Mulinia lateralis*, in a 48-hour exposure to effluents and receiving waters. The sub-lethal endpoint of the test is larval shell formation. This method was adapted at EPA's Environmental Research Laboratory at Narragansett, RI from existing bivalve test methods (Standard Methods, 1976; ASTM, 1989).

II. NECESSARY MATERIALS AND EQUIPMENT

A. Clam Cultures

It is preferable to obtain embryos from an in-house culture unit. If it is not feasible to culture adult clams, adult spawning stock can be collected from the field or purchased from commercial supply houses. Adults can be shipped wrapped in towels moistened with seawater in an insulated container.

- Aquaria--5 gallon, enough to hold the desired number of clams at the desired range of salinities.
- Uncontaminated saline culture water--natural seawater or hypersaline brine made from natural seawater (see SOP--Making Hypersaline Brine) and diluted with deionized water.
- Plastic buckets or waste baskets--about 15 L capacity, for rearing embryos to juvenile clams.
- Uncontaminated air supply with gang valves and air stones--for aerating embryo and adult clam cultures.
- Nitex screens--opening sizes 36, 53, 74, and 125 μ m to screen down larval clam cultures.
- Environmental chamber or equivalent facility with temperature control ($24^{\circ}\text{C} \pm 1^{\circ}\text{C}$)--for holding tanks containing adult brood stock and juvenile clam cultures.

ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST

ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 2 OF 18

- Water purification system--deionized water (DI) or equivalent.
- Dissecting microscope--for monitoring growth of larval clam cultures.
- Refractometer--for determining salinity of culture tanks.
- Thermometers, laboratory grade--for monitoring temperatures in clam culture tanks.

B. Algal Cultures

- Uncontaminated natural seawater culture water.
- Polycarbonate carboys--20 L capacity, for culturing the algae *Isochrysis galbana* and *Tetraselmis suecica* for use as food for culture animals.
- Environmental chamber or equivalent facility with temperature control (18°C - 20°C) and uncontaminated air supply--for culturing algae.
- Nutrients for algal cultures-- NaNO_3 , NaH_2PO_4 , EDTA, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$.
- Uncontaminated air supply--for aerating algal cultures.
- Disposable plastic or glass pipettes--10 mL capacity, for delivering air to algal cultures.
- Silastic tubing--for delivering air to algal cultures.
- Water purification system--deionized water or equivalent.

C. Toxicity Tests

- Environmental chamber or equivalent facility with temperature control ($22 \pm 2^\circ\text{C}$)--for incubating test.
- Refrigerator (4°C)--for preparing adults to spawn.
- Water purification system--deionized water (DI) or equivalent.
- Count register, 2-place--for recording larvae shell/no shell counts.
- Sedgwick-Rafter counting chamber--for counting embryo stock and larvae.
- pH meter--for measuring the pH of effluent samples.
- Dissecting microscope--for counting embryo stock.
- Compound microscope--for verifying egg fertilization and counting larvae.
- Refractometer--for determining salinity of samples.
- Thermometers, laboratory grade--for measuring sample temperatures and monitoring spawning temperature.
- Beakers or Erlenmeyer flasks, glass or non-toxic plasticware--for mixing dilution water.
- Small (100-200 ml) crystallization or culture dishes, or equivalent--for spawning adults.
- Pipets, adjustable--0.1 to 0.2 ml, 0.5 ml, 1.0 ml, 5.0 ml with disposable tips--for dispensing embryos and mixing test solutions.
- Pasteur pipets--to sample dilutions for salinity checks.
- Markers, water-proof--for marking containers.
- 72 um Nitex screen--for screening embryo stock.
- Forceps, non-toxic plastic--for removing spawning males.
- Data sheets (one set per test)--for data recording (Figure 1).
- Formalin, 100%--for killing/preserving larvae.
- Scintillation vials, 20 ml glass, disposable, with caps--for use as test vessels.
- Tape, colored--for labelling test boxes.
- Buffer, pH7, (or as per instructions of instrument manufacturer)--for standards and

ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST

ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 3 OF 18

calibration check.

- Reference toxicant solutions.
- Effluent or surface water to be tested--the volume of effluent required is about 100 mL. Samples should be tested while less than 24 hr (maximum of 48 hr) old.
- Uncontaminated saline test dilution water--natural seawater or hypersaline brine made from natural seawater (see Brine SOP) and diluted with deionized water.

III. METHODS

A. Clam Cultures

Adult clams for use as brood stock may be obtained from sediment collections (dredge, grab samples, etc.) from bays and estuaries along the Atlantic and Gulf of Mexico coasts from Malpeque Bay, Canada to northeastern Mexico and in the West Indies (Rhodes et al., 1972). Coot clams have been found at salinities ranging from 1.4 to 71.5‰ (Breuer, 1957). The species generally is found in soft (mud/clay) sediment but is occasionally found in surf-stirred sand (Smith, 1964). Brood stocks may also be obtained from commercial supply houses or by rearing laboratory spawned animals to maturity. It is recommended that laboratory brood stocks be replaced annually with feral stock to minimize inbreeding.

1. *Mulinia lateralis* can be continuously cultured in the laboratory from embryos to adults. Embryos are obtained as described in 'Toxicity Tests'.
2. Hold embryos and developing larvae at $24^{\circ} \pm 2^{\circ}$ C in 15 liter plastic tanks with gentle aeration at concentrations up to 15,000/liter.
3. The early embryo and larvae cultures should be maintained within ± 5 ppt salinity of the parent stock. The salinity can be adjusted at a rate of 2 ppt per day if desired.
4. Screen the larvae and replace the culture water at least three times per week. Nitex screens with opening sizes of 36, 53, 74, and 125 microns, depending on the age of the cultures, are used to collect the larvae.
5. Larval cultures are fed, ad libitum, the microalga *Isochrysis galbana* (see 'Algal Cultures') daily. Under the above conditions the larvae should metamorphose in approximately two weeks.
6. Newly metamorphosed juveniles are transferred to 5 gallon glass aquaria containing 2-3 cm of fine sand covered by two to three gallons of gently aerated seawater. The aquaria are held at $24^{\circ} \pm 2^{\circ}$ C and at salinities ranging from 10 to 35 ± 2 ppt.
7. Two liters of the aquarium water are removed daily and replaced with one liter each of *Isochrysis galbana* and *Tetraselmis suecica* cultures. This daily feeding also provides sufficient water replenishment necessary for maintaining healthy laboratory populations. If a brood stock is being maintained at a reduced salinity, it may be

necessary to add appropriate amounts of deionized water to compensate for the salinity of the algae cultures (approximately 30 ppt).

8. After the juveniles grow to a visible size (4-5 mm), they should be culled to about 150-200 animals per aquarium.
9. *Mulinia lateralis* juveniles held at 24° C should become sexually mature after approximately 60 days.
10. At least five reference toxicant tests should be performed using new cultures in order to verify the quality of the embryos obtained.
11. The clams can be spawned and the embryos used in toxicity tests at salinities within ± 5 ppt of the holding salinity. Therefore, three brood stocks held at 10, 20, and 30 ppt salinity respectively, can provide embryos for testing at salinities ranging from 10 (the lowest salinity recommended) to 35 ppt. Otherwise, brood stocks can be acclimated to higher or lower salinities at a rate of 5 ppt per day. Clam cultures should be held at the desired salinity for at least one week before use in toxicity testing.

B. Algal Cultures

Isochrysis galbana and *Tetraselmis suecica* can be cultured in 20 liter polycarbonate carboys that are normally used for bottled drinking water.

1. Filtered seawater (one micron) is added to the carboys and the autoclaved (250° C for 35 minutes).
2. After cooling to room temperature, the carboys are placed in a controlled temperature chamber at 18-20° C.
3. One liter of starter culture of one algal species and 200 ml of nutrients are added to each carboy.
4. The algal cultures are vigorously aerated via a pipette inserted through a foam stopper at the top of the carboy.
5. A dense algal culture should develop in 7-10 days and should be used by day 17.
6. Start-up of cultures should be made two to three times a week. One carboy each of *I. galbana* and *T. suecica* will provide enough food for six aquaria for three days. The total number of carboys in use will depend upon the number of aquaria and larval cultures being fed.
7. Formula for algal culture nutrients

-Add 180 gm NaNO₃, 12 gm NaH₂PO₄, and 6.16 gm EDTA to 12 liters of

ERL N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST

ERL N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 5 OF 18

deionized water. Mix with a magnetic stirrer until all the salts are dissolved (at least one hour).

-Add 3.78 gm $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and stir again. The solution should be a bright yellow color.

C. Toxicity Tests

1. Test Solutions--Surface Waters

a. Surface water toxicity is determined with samples used directly as collected, except when salinity adjustment is necessary. If effluent and surface water tests are conducted concurrently, the salinities of these tests should be similar. The test salinity should not be more than 5‰ higher or lower than the adult holding salinity, and must be in the range of 10 to 30‰.

2. Test Solutions--Effluents

a. The selection of the range of effluent test concentrations should be based on the objectives of the study. If an effluent is of unknown toxicity and 100‰ hypersaline brine (HSB, see Brine SOP) is the diluent, seven effluent concentrations may be tested. The highest effluent concentrations possible (for effluents with no salinity) ranges from 70% at 30‰ to 90% at 10‰. If the effluent is known or suspected to be highly toxic, a lower range of concentrations should be used.

b. Just prior to testing, the temperature of the sample should be adjusted from the holding temperature (4C) to $22 \pm 2^\circ\text{C}$ and the pH should be adjusted to 7.25 to 8.25 (7.75 is optimal for larval development (Calabrese, 1971)).

c. Test dilution water may be natural seawater, receiving water, or HSB prepared from natural seawater. Prepare 3 L of dilution water for spawning the adults, diluting the effluent, and for the test control. Natural seawater and local waters may be used as additional controls.

d. Test solution preparation and test exposure may be performed in the box in which scintillation vials are shipped. Twenty-seven disposable glass scintillation vials are arranged in nine rows of three vials each (for two controls and seven effluent concentrations) (Figure 2). The vials should be labeled with the effluent name and test concentration.

e. Except for the three replicates of the high concentration, 10 mL of control or diluent solution at the test salinity should be added to each vial.

f. The control vials should be capped during effluent addition and mixing to prevent contamination.

g. Appropriate volumes of HSB and effluent should be added to the three vials of

the high concentration to total 20 mL of solution at the desired effluent concentration and salinity. For example, if the high concentration is to be 80% effluent and the test salinity is 20‰, 4 mL of 100‰ HSB and 16 mL 0‰ effluent should be added to each vial of the high concentration for a total of 20 mL.

h. Mix the high concentration by using a 5 mL pipet to slowly draw up a 5 mL aliquot from the vial and then dispense it back into the vial. Repeat this action 3-4 times.

i. The salinity of each replicate should then be checked to assure that the proper volumes of effluent and HSB were added and that the solution is thoroughly mixed.

j. Transfer 10 ml from each high concentration replicate to the corresponding replicate of the next lowest concentration (which should already contain 10 mL of diluent).

k. Mix these solutions thoroughly and transfer 10 mL from each replicate to the corresponding replicate of the next lowest concentration. Repeat the mixing and transferring through the lowest test concentration.

l. After the last solutions are mixed, transfer 10 mL from each vial containing the lowest concentration solution into a container marked "WASTE". There should be 10 mL of solution in each test vial.

m. The test box should be labeled with colored tape and a water-proof marker to indicate test species, test date, and the effluent being tested.

3. Obtaining Embryos for Toxicity Tests

a. Select about 20 fertile adults from laboratory cultures maintained within 5‰ of test salinity. Fertile animals can usually be identified by pale pink or peach coloration in the umbo region.

b. Rinse the animals in clean seawater and place them in a small crystallization dish. All animals should be lying on the bottom of the dish without touching other animals.

c. Cover the animals with clean natural seawater or seawater prepared with HSB at the salinity of the clam culture.

d. The dish should then be placed in a 4°C refrigerator for 0.5 to 2 h.

e. After cooling, the animals should be thoroughly rinsed several times in 25-28°C autoclaved seawater or HSB seawater diluent.

f. Animals are then barely covered with autoclaved seawater or HSB seawater and warmed to 25-28°C. Spawning temperature should not exceed 29°C, or damage could result to the embryos (Kennedy et al., 1974).

**ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST**

ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 7 OF 18

- g. The animals should begin to spawn shortly after being warmed. Sperm may be identified by a white, milky appearance, while eggs will appear pinkish and of a more granular texture.
- h. Spawning males should be removed after a few seconds (before the spawning water becomes cloudy) and placed into another dish containing clean diluent. The exact concentration of sperm is not critical; however, the presence of excessive sperm should be avoided.
- i. Rarely, it may be necessary to strip clams in order to obtain gametes, although natural spawning of adults is preferable:
1. Remove one valve from an adult clam and, in a small glass dish containing clean seawater about 0.5 cm in depth, gently macerate the gonad with a glass rod or pipette.
 2. Examine the contents of the dish under 100x magnification to verify the presence of normal eggs or active sperm.
 3. Sacrifice several animals until sufficient viable eggs and sperm have been obtained.
 4. Mix the gametes together in a clean beaker.
- j. Swirl the dish to suspend the eggs and to mix the eggs and sperm.
- k. Pour the contents of the dish through a seawater-rinsed 72 μ m Nitex mesh screen into a small (100 mL) beaker to remove large debris. It is not necessary to rinse the screen into the beaker, since the roughly 50 μ m eggs (Rhodes et al., 1972) will pass through the screen.
- l. Verify that the coot clam eggs are fertilized by examining them microscopically at 100x magnification. Fertilized eggs are readily observable; they are spherical, even in color, and possess a polar body (small transparent bump on egg).
- m. If fertilization is in doubt (eggs are irregularly shaped, unevenly shaded), allow the eggs to incubate for 40-50 minutes to allow first cleavage to occur.
1. If all of the eggs are not fertilized, add a small amount of sperm from the dish containing the spawning males and swirl the stock to mix the gametes.
 2. Wait about 10 minutes, then re-examine the eggs to verify that fertilization has taken place.
 3. Embryos must be added to the test when less than 2 hr old.
- n. Swirl or stir the stock solution to evenly suspend the embryos, then sample 0.5

ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST

ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 8 OF 18

mL and dispense into a scintillation vial containing 9.5 mL seawater or HSB diluent at the spawning salinity.

o. Cap the vial and invert it several times to mix the sample.

p. Immediately sample 1.0 mL from this vial and dispense into a Sedgwick-Rafter counting chamber.

q. Count the entire contents of the chamber under a dissecting scope at 24x magnification. At the desired embryo stock concentration the number of embryos in the chamber should be between 188 and 375.

r. Multiply this count by 20 to determine the number of embryos per mL of the stock solution. The desired embryo concentration is between 3,750 and 7,500 embryos per mL.

1. If the number of embryos is less than 3,750 per mL, allow the embryos to settle undisturbed for 10-15 minutes, then carefully concentrate the stock solution by pipetting water from the top of the solution. If the remaining volume of embryo stock is insufficient to perform the test, more adults must be spawned.

2. Dilute the embryo stock with additional control water if the stock is more concentrated than 7,500 embryos per mL.

3. Once the stock solution has been concentrated or diluted, repeat the counting procedure.

s. Each test vial should contain 750 embryos (75 embryos/mL test solution).

1. To determine the amount of embryo stock to add to each vial, divide 750 by the number of embryos per mL in the stock solution. Between 0.100 and 0.200 mL embryo stock should be added to each vial.

2. Add the same volume of stock to each vial, swirling or stirring the solution before each addition to keep the embryos evenly distributed in the stock.

t. Following embryo addition, cap the vials and place them in a temperature controlled room or incubator at $22 \pm 2^{\circ}\text{C}$ for 48 h.

4. Terminating the Test

a. The test is terminated after 48 h of exposure.

b. Terminate and preserve the test by the addition of 0.5 mL 100% formalin to each vial. CAUTION: This procedure should be performed under a fume hood.

**ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST**

ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 9 OF 18

- c. Re-cap the vials after all of the replicates have been preserved.
- d. The test may be evaluated immediately or stored for up to one week.
- e. To determine the presence of shells, transfer 1 mL of larvae and solution from the bottom of a test vial to a Sedgwick-Rafter counting chamber.
- f. Observe the larvae using a compound microscope at 100x magnification.
- g. Count about 100 larvae per sample, and record the number counted and the number without complete shells. Some larvae may have complete but abnormal shells--these are recorded as having shells. CAUTION: Because the samples are fixed in formalin, a ventilation hood is set up surrounding the microscope to protect the analyst from exposure to fumes.
- h. Tests are acceptable if 60% or greater of control larvae have shells.

IV. TROUBLE SHOOTING

- 1. Toxic substances may be introduced by contaminants in dilution water or on hands, glassware, sample hardware, and testing equipment. Be sure that all materials which will be in contact with test materials or animals are thoroughly clean and, if necessary, soaked in seawater.
- 2. Adverse effects of low dissolved oxygen concentrations, high concentrations of suspended and/or dissolved solids, and extremes of pH, may alter the effects of toxic substances. If these conditions exist, this should be noted on datasheets.
- 3. Improper effluent sampling and handling may adversely affect test results.
- 4. A single toxicity test or group of toxicity tests should be performed and scored by the same person, since variations in these activities may exist among researchers.

V. STATISTICAL ANALYSIS AND DATA USAGE

- 1. Tabulate and summarize the data. Calculate the percent of larvae with no shell for each replicate.
- 2. The endpoints of toxicity tests using the coot clam are based on the reduction of percent of larvae with shells. Point estimates, such as EC1, EC5, EC10, and EC50, are calculated using Probit or Spearman-Kärber analysis (Finney, 1971; Hamilton, Russo, and Thurston, 1977). A hypothesis test approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981), is used to estimate NOEC and LOEC values. See the appendices of

EPA/600/4-87/028 for examples of the manual computations, program listings, and examples of data input and program output.

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ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST

ERL-N SOP 1 03.008
REVISION 0
DECEMBER 1990
PAGE 11 OF 18

Figure 1. Sample data sheet.

Mulinia lateralis

48 HOUR EMBRYO-LARVAL TOXICITY TEST

TEST ID:	STUDY:
PERFORMED BY:	TOXICANT:
TIME TEST BEGUN:	TIME TEST ENDED:
CULTURE SALINITY:	TEST SALINITY:
EFFLUENT SALINITY:	EFFLUENT pH:
TEST CONTROL:	DATE SAMPLE COLLECTED:

Embryo Stock: 3,750 - 7,500/ml desired

Dilute 0.5 ml original stock in 9.5 ml culture salinity seawater.
Count 1.0 ml of dilute embryos in Sedgwick-Rafter cell.

Initial Count: $C_1 =$
Stock Concentration: $20 \times C_1 = S_1 =$ embryos/ml

If the volume or concentration of the original stock is adjusted,
repeat counting procedure:

Final count: $C_2 =$
Final Stock Conc.: $20 \times C_2 = S_2 =$ embryos/ml

To determine the volume of stock to add to each test vial:

Volume: $(750 \text{ embryos})/S^1 =$ ml

Volume should be between 0.100 and 0.200 ml.

Additional Comments:

**ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST**

**ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 12 OF 18**

ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST

ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 13 OF 18

Mulinia lateralis 48 HOUR E/L TOXICITY TEST

VERIFICATION:

TEST ID:

STUDY:

TOXICANT:

LABORATORY CONTROL:

SITE CONTROL:

SAMPLE COMMENT:					
SAMPNUM:	TYPE:	CONC:	REP	CTD	NO SHELL
COMMENTS:			1	100	
			2	100	
			3	100	

SAMPLE COMMENT:					
SAMPNUM:	TYPE:	CONC:	REP	CTD	NO SHELL
COMMENTS:			1	100	
			2	100	
			3	100	

SAMPLE COMMENT:					
SAMPNUM:	TYPE:	CONC:	REP	CTD	NO SHELL
COMMENTS:			1	100	
			2	100	
			3	100	

SAMPLE COMMENT:					
SAMPNUM:	TYPE:	CONC:	REP	CTD	NO SHELL

ERL-N STANDARD OPERATING PROCEDURE
 COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
 TOXICITY TEST

ERL-N SOP 1.03.008
 REVISION 0
 DECEMBER 1990
 PAGE 14 OF 18

COMMENTS:	1	100	
	2	100	
	3	100	

VERIFICATION:

TEST ID:

STUDY:

TOXICANT:

SAMPLE COMMENT:					
SAMPNUM:	TYPE:	CONC:	REP	CTD	NO SHELL
COMMENTS:			1	100	
			2	100	
			3	100	

SAMPLE COMMENT:					
SAMPNUM:	TYPE:	CONC:	REP	CTD	NO SHELL
COMMENTS:			1	100	
			2	100	
			3	100	

SAMPLE COMMENT:					
SAMPNUM:	TYPE:	CONC:	REP	CTD	NO SHELL
COMMENTS:			1	100	
			2	100	
			3	100	~

ERL-N STANDARD OPERATING PROCEDURE
 COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
 TOXICITY TEST

ERL-N SOP 1.03.008
 REVISION 0
 DECEMBER 1990
 PAGE 15 OF 18

SAMPLE COMMENT:			
SAMPNUM:	TYPE:	CONC:	
COMMENTS:			REP
			CTD
			NO SHELL
			1
			2
			3

ERL-N STANDARD OPERATING PROCEDURE COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL TOXICITY TEST

ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 16 OF 18

COMMENTS

Adults cooled: _____

Adults spawned: _____

Embryos added: _____

Exposure ended: _____

Test read:_____

Data analyzed: _____

Ref. Tox. EC50: _____

Data reported: _____

This image shows a single sheet of white paper with horizontal ruling lines. The lines are evenly spaced and run across the width of the page. There are no margins, text, or other markings on the paper.

ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST

ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 17 OF 18

FIGURE 2. SAMPLE *Mulinia lateralis* EFFLUENT TEST BOX. THIS SAMPLE TEST USES HYPERSALINE BRINE DILUTED WITH DEIONIZED WATER (HB+DI) AS THE DILUENT AND TEST CONTROL AND IS TO BE CONDUCTED AT 20‰. A NATURAL SEAWATER CONTROL (NAT.SW) IS ALSO BEING TESTED. THE EFFLUENT TESTED IS 0‰ (EF.). THE ROWS ON THE LEFT SHOW THE TEST SET-UP PRIOR TO EFFLUENT MIXING; THE ROWS ON THE RIGHT SHOW A MIXED TEST READY FOR EMBRYO ADDITION. ALL OF THE VIALS ON THE RIGHT SHOULD CONTAIN 10 ML OF TEST SOLUTION.

UNMIXED TEST

	HB+DI 10mL	HB+DI 10mL	HB+DI 10mL	
	NAT.SW 10mL	NAT.SW 10mL	NAT.SW 10mL	
	HB+DI 10mL	HB+DI 10mL	HB+DI 10mL	
	HB+DI 10mL	HB+DI 10mL	HB+DI 10mL	
	HB+DI 10mL	HB+DI 10mL	HB+DI 10mL	
	HB+DI 10mL	HB+DI 10mL	HB+DI 10mL	
	HB+DI 10mL	HB+DI 10mL	HB+DI 10mL	
	HB+DI 10mL	HB+DI 10mL	HB+DI 10mL	

MIXED TEST

	HB+DI 0% EF.	HB+DI 0% EF.	HB+DI 0% EF.	
	NAT.SW 0% EF.	NAT.SW 0% EF.	NAT.SW 0% EF.	
	1.3% EF.	1.3% EF.	1.3% EF.	
	2.5% EF.	2.5% EF.	2.5% EF.	
	5.0% EF.	5.0% EF.	5.0% EF.	
	10.0% EF.	10.0% EF.	10.0% EF.	
	20.0% EF.	20.0% EF.	20.0% EF.	
	40.0% EF.	40.0% EF.	40.0% EF.	

**ERL-N STANDARD OPERATING PROCEDURE
COOT CLAM (*Mulinia lateralis*) EMBRYO/LARVAL
TOXICITY TEST**

ERL-N SOP 1.03.008
REVISION 0
DECEMBER 1990
PAGE 18 OF 18

	4mL HB 16mL EF.	4mL HB 16mL EF.	4mL HB 16mL EF.	
--	-----------------------	-----------------------	-----------------------	--

	80.0% EF.	80.0% EF.	80.0% EF.	
--	--------------	--------------	--------------	--

**ERL-N STANDARD OPERATING PROCEDURE
MICROTOX**

**ERL-N SOP 1.03.009
REVISION 0
MARCH 1991
PAGE 1 OF 1**

POINT OF CONTACT:

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I. OBJECTIVE

This method determines acute toxicity in effluent and receiving waters using Beckman's Microtox™ System. The endpoint of this method is based on changes in the light produced by bioluminescent bacteria.

II. NECESSARY MATERIALS AND EQUIPMENT

- Beckman's Microtox Model 2055 Toxicity Analyzer and associated necessary equipment (listed in operating manual)

III. METHODS

The methods performed at ERL-N to determine acute water column or effluent toxicity using Microtox are widely used. These methods are specific to the use of the Microtox system and are provided to the purchaser of the system by the manufacturer. These methods must be followed carefully in order to properly determine acute toxicity.

IV. TROUBLE SHOOTING

Discussed in operating manual.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Data are used along with other toxicity tests in assessing the toxicity of an effluent or receiving water.
2. Statistical analyses are discussed in the operating manual.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
PERFORMING THE PHAGOCYTOTIC INDEX AND
KILLING ABILITY ASSAY**

ERL-N SOP 1.03.010
REVISION 0
DECEMBER 1990
PAGE 1 OF 4

POINT OF CONTACT:

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I. OBJECTIVE

This SOP describes an *in vitro* method referred to as the Phagocytic Index and Killing Ability (PIKA) assay. This test is designed to quantify the level of activity, capacity, and bactericidal efficiency of phagocytic cells from fish and bivalves.

II. NECESSARY MATERIALS AND EQUIPMENT

- Spectrophotometric cuvettes
- Syringes (1, 3, and 10 cc) with 20 gauge needles
- Tissue culture slides (eight well)
- Polypropylene test tubes (20 mL) with caps
- Petri dishes
- Filter pads
- Coverslips (24x60 mm)
- Paraffin wax
- Small cotton swabs
- Paper towels
- Dissecting implements
- Pipetmen (200 ul, 1000 ul, and 5000 ul) with sterile tips
- Laminar flow hood
- Spectrophotometer
- UV microscope with 35 mm camera
- Bunsen burner
- Hemocytometer
- Cell counter
- Light microscope
- Tube rack
- Shaker table
- Incubator (with chilling cap.)
- Sterile seawater
- Leibowitz-15 culture media (L-15)
- Streptomycin
- Penicillin
- Dextrose
- Gay's balanced salt solution
- Oxidase reagent

**ERL-N STANDARD OPERATING PROCEDURE
PERFORMING THE PHAGOCYTOTIC INDEX AND
KILLING ABILITY ASSAY**

ERL-N SOP 1.03.010
REVISION 0
DECEMBER 1990
PAGE 2 OF 4

- Vibriostat 129
- Bovine serum
- Bovine fibronectin
- 95 % ethyl alcohol
- Brain heart infusion agar (BHI)
- TCBS agar
- MS222
- Trypan blue 4 % solution
- Acridine orange staining solution
- Crystal violet staining solution

III. METHODS

A. Flounder PIKA Assay (Daniels 1988)

1. Anesthetize flounder with MS222 in seawater at a concentration of 10 mg/L seawater.
2. Insert a 10 mL syringe into the caudal vein and remove as much blood as possible.
3. Swab the abdominal surface on the pigmented side liberally with 95 % ethyl alcohol and remove abdominal side in flow hood using sterile technique.
4. Remove the head kidney tissue anterior to the fourth vertebra (posterior edge of the pronephros as determined by histological section) and place in 5 mL of cold L-15 medium supplemented with glucose (0.33 g/L), streptomycin (0.06 g/L) and penicillin (0.1 g/L).
5. Centrifuge pronephros suspension for 10 min. at 300 x g and resuspend in 2 mL of glucose supplemented L-15.
6. Homogenize with a hand-held homogenizer on ice for 2 minutes and resuspend in 2 mL of glucose-supplemented L-15.
7. Determine the total number of leucocytes/mL with a Neubaur hemocytometer. Macrophage density is calculated as 10% of the total leucocyte count. Leucocyte viability is determined using 4% trypan blue.
8. Normalize the density of macrophages to a final concentration of 10^6 cell/mL of L-15 supplemented with glucose and 10% serum and allow to stand for 30 minutes.
9. Prepare a bacteria suspension, determine its absorbance, and use a standard curve to estimate concentration. Adjust the volume of bacteria suspension to obtain a concentration of 5×10^7 cells/mL in glucose-supplemented L-15 and 10% serum. Allow the bacteria suspension to stand for 15 min. at 15° C prior to use.

Note: Maintain *Vibrio alginolyticus* cultures on BHI (Difco) containing 1% sodium

**ERL-N STANDARD OPERATING PROCEDURE
PERFORMING THE PHAGOCYTOTIC INDEX AND
KILLING ABILITY ASSAY**

ERL-N SOP 1.03.010
REVISION 0
DECEMBER 1990
PAGE 3 OF 4

chloride at room temperature. Harvest cultures after seventy-two hours and resuspend in 2 mL of Gay's balanced salt solution (Gibco). The identification of the cultured bacteria should be periodically confirmed by culturing on TCBS agar and testing with oxidase reagent (Marion Scientific) or Vibriostat 129 (Sigma Chemical). A standard curve for estimating suspension concentration should be prepared using a quantitative plating method and turbidity determinations (Benson, 1969).

10. Mix 1 mL macrophage suspension with 1 mL of bacteria suspension and dispense 0.5 mL portions in two wells of a cell culture slide (Thomas Scientific) pre-coated for 1 hour with bovine fibronectin (Sigma Chemical). Reserve a third 0.5 mL portion of macrophage suspension on a tissue culture slide with 10% seawater added as a control.

12. Incubate for 120 min. at 15°C in a moist sterile chamber.

13. Wash the tissue culture slides with fresh L-15 leaving only adherent macrophages.

14. Stain the monolayer of macrophages with acridine orange solution (14 mg/100mL) for 1 min., wash with L-15, stain with crystal violet solution (50 mg/mL) for 1 min. and wash again with L-15.

15. Coverslip the slide and seal edges with hot wax.

16. Using a fluorescent microscope at 900-1200x, count the number of phagocytic cells, the number of green and the number of red intracellular bacteria observed among 100 cells. The phagocytic activity index (PAI), equal to the percentage of phagocytic cells, is calculated by dividing the number of active phagocytes by the total number of cells observed. The phagocytic capacity or phagocytic index (PI) can be calculated by dividing the total number of intracellular bacteria (red and green) by the number of phagocytes counted. The killing ability index (KI) or bactericidal efficiency is calculated by dividing the number of dead (red) intracellular bacteria by the total number of intracellular bacteria (red and green) counted.

B. Bivalve PIKA Assay (Daniels, unpublished)

1. Withdraw hemolymph (0.2 mL) from the pericardial area with a sterile 3 mL syringe containing 1.8 mL sterile seawater and equipped with a 20 gauge disposable needle.

2. Quantify the number of hemocytes with a hemocytometer.

3. Place three 0.5 mL portions of the hemocyte suspension from each animal in three wells of a sterile culture slide.

4. Add a volume of challenge bacteria suspension to the hemocyte suspension (Prepared as above) with a concentration 5 times the hemocyte concentration into two of the three wells. The third well receives a 10% volume of seawater instead of bacteria suspension and serves as a control.

Note: It is important to mix a bacteria suspension with an absorbance (ABS) near 0.7

**ERL-N STANDARD OPERATING PROCEDURE
PERFORMING THE PHAGOCYTOTIC INDEX AND
KILLING ABILITY ASSAY**

ERL-N SOP 1.03.010
REVISION 0
DECEMBER 1990
PAGE 4 OF 4

ABS at 560 nm. Volume adjustments made at this high concentration of bacteria should minimize to less than 10% of the difference in total volume between wells.

8. Incubate for 60 minutes (agitate slowly for 30 min. on shaker table) at 20°C.
9. Following incubation, rinse the cell cultures with sterile seawater and sequentially stain with acridine orange and crystal violet, coverslip, and count as described above.

IV. TROUBLE SHOOTING

Included in Methods Section.

V. STATISTICAL ANALYSIS AND DATA USAGE

Results of PIKA assays are analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's Procedure. Data are used in assessments of risk to marine and estuarine sites.

VI. REFERENCES

- Benson, H.J. 1976. Bacterial Population Counts. In: Microbiological Applications (Short Edition). WM. C. Brown Co. Pub., Dubuque, Iowa, pp. 71-16.
- Daniels, T.G. 1988. Development of a Fluorescent *In Vitro* Method to Test the Phagocytic Engulfment and Intracellular Bactericidal Abilities of Macrophages from Winter Flounder, *Pseudopleuronectes americanus* (Walbaum). M.S. Thesis, University of Rhode Island, Kingston, RI. xi + 105 pp.

POINT OF CONTACT:

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I. OBJECTIVE

In accordance with the Marine Protection, Research, and Sanctuaries Act of 1977, all proposed operations involving the transportation and dumping of dredge material into ocean waters, must be evaluated to determine the potential environmental impact of such activities (EPA, 1977). As part of the regulatory process, solid phase bioassays are conducted with sensitive benthic marine organisms to provide information concerning the bioaccumulation potential and toxicity associated with dredge material. The following document defines the standard operating procedures used in conducting solid phase bioaccumulation testing.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Construction of Aquaria and Head Trough

- Standard 10 gallon (37.8 L) glass aquaria with covers
- Silastic tubing (3/16 inch I.D. x 5/16 inch O.D.)
- Capillary tubing (1 mm)
- 2 oz. glass jars
- 1/2 inch nylon T-connectors
- 74 μ m Nytex screening

B. Collection and Preparation of Sediment

- Polyethylene holding containers (35 and 55 gallon)
- Stainless steel sieves (1 and 2 mm)
- Shovel
- Sediment scoops
- Smith-MacIntyre grab sampler, caulking bar, stand, weights, collection bucket
- Press sieving paddles
- 10 gallon holding containers with lids

C. Test Conduct

- Thermometer
- Refractometer
- Nester dissolved oxygen meter and BOD probe
- Log book

**ERL-N STANDARD OPERATING PROCEDURE
SOLID PHASE BIOACCUMULATION TESTING**

ERL-N SOP 1.03.011
REVISION 0
DECEMBER 1990
PAGE 2 OF 4

- Glass sampling jars
- Tweezers

III. METHODS

A. Construction Of Aquaria and Head Trough

1. Standard 10 gallon glass aquaria are used in solid-phase bioaccumulation testing. Tanks are modified with 1/2 inch nylon T connectors to allow water to leave each tank. Nylon T's, with a small piece of silastic tubing, are inserted into a pre-cut hole, located in the center and approximately 1 inch down from the end of each tank.
2. Covers for tanks are constructed of 1/8 inch plexiglass, with a 1/2 inch hole drilled in the center to allow silastic tubing to pass through to each tank.
3. Using a nylon clip, a piece of nitex screening (74 μ m mesh size) is secured around the outport hole of each tank to prevent any loss of animals.
4. Filtered sea water enters each tank from a head trough above, which is constructed of PVC piping with 1/4 inch nipple fittings threaded at 8 inch intervals.
5. Silastic tubing (3/16 I.D. x 5/16 O.D.) is attached to each barbed nipple fitting located on the head trough and a 1 mm capillary bore is placed on the end to regulate the flow of water into each tank.

B. Collection and Preparation of Sediment

1. Sediment is collected using a Smith-MacIntyre grab sampler and pressed sieved using paddles through a 2 mm screen into clean 10 gallon holding containers. All contaminated sediments should be pressed sieved in the field prior to returning.
2. After sieving, sediment is transferred into 35 gallon polyethylene barrels with covers and transported back to ERL-N and stored at 4°C in the outside cooler until testing. Contaminated sediments should be placed in approved DOT shipping containers prior to transport.
3. Chain of custody procedures should be followed at all times once the sediment has arrived at ERL-N. This includes documenting any transfer of sediment or change to the contents prior to conducting the actual test.

C. Test Conduct

1. Prior to initiating the test, sediment is removed from the outside cooler and allowed to equilibrate to room temperature.
2. After 3-4 hours, sediments are thoroughly homogenized using a shovel for a period of 15-20 minutes.

3. Sediment is loaded into aquaria to depth of 5 cm and the flow of seawater started to each tank at a rate of 200 ml/min. The flow of water should be diverted into a 2 oz. jar located on the bottom of each tank to prevent sediment from being resuspended. All tanks are allowed an acclimation period of 24 hours before any animals are added.

4. Background sediment and tissue samples should be taken just prior to animals being added to tanks, and should consist of at least 3 replicates from each treatment and control.

5. Appropriate benthic marine organisms used in conducting solid-phase bioassays can be found in appendix F in the Implementation Manual entitled: "Ecological Evaluation of Proposed Discharge of Dredged Material into Ocean Waters." The selection of animals should include at least one species each representing filter-feeding, deposit-feeding, and burrowing species.

6. After a 24 hour acclimation period, benthic organisms are removed from holding aquaria and counted into glass finger bowls and examined for healthiness prior to placement into tanks. All animals are counted twice before placement into appropriate aquaria. Any animals showing signs of stress (i.e. lack of burrowing) should be removed after a 30 minute period and replaced.

7. Water quality measurements such as temperature, salinity, and dissolved oxygen are recorded daily. Mortality is also recorded and any dead animals are removed from tanks.

8. Bioaccumulation testing is usually conducted at 20°C, with salinity ranging from 28-32 parts per thousand. Dissolved oxygen should be maintained at or above 60% saturation throughout the course of the experiment.

9. Upon termination of the test (usually after a 10 exposure), flow of seawater is stopped and overlying water is siphoned from each of the tanks. The remaining sediment is sieved over a 2 mm screen and animals are gently removed, washed, and placed in finger bowls where they are counted.

10. Animals are placed in clean tanks with flowing sea water to allow for a 24 hr gut purge. Following gut purging, animals are removed, rinsed, and placed in muffled jars for chemical analysis.

IV. TROUBLE SHOOTING

A. Test Conduct

1. Removal of animals from holding tanks should be done in such a way as to minimize handling and avoid injury to the animal. Attention should be given to transferring and holding animals no longer than is necessary, as some animals (i.e. worms) will inflict harm on one another.

2. In order to maintain constant flow rates throughout the course of the experiment, capillary tubes should be checked daily for any obstructions and cleaned at least once every week.
3. Head troughs should also be maintained, making sure algal growth does not impede the flow of water through nipple fittings.
4. Dissolved oxygen concentration should always be maintained above 60% saturation. If levels fall below 60% saturation, airstones should be placed in each tank to supplement dissolved oxygen concentrations. Only a slight amount of bubbling is necessary, as to minimize any resuspension of sediment.

V. STATISTICAL ANALYSIS AND DATA USAGE

Data are statistically analyzed using one-way analysis of variance (ANOVA) procedures using any of the multiple comparison tests (ie- Duncan's, Dunnett's procedures (Duncan 1955; Dunnett 1955)). Comparisons are usually made between the control and any of the treatments based on bioaccumulation levels of various analytes. Statistical comparisons are also made between the control and any of the treatments regarding mortality as well.

VI. REFERENCES

- Duncan, D.B. 1955. Multiple range and multiple F tests. *Biometrics* 11:1-42.
- Dunnett, C.W. 1955. A multiple comparisons procedure for comparing several treatments with a control. *JASA* 50:1096-1101.
- U.S. EPA, 1977. Environmental Protection Agency/Corps of Engineers Technical Committee on Criteria for Dredged and Fill Material into Ocean Waters.

POINT OF CONTACT:

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I. OBJECTIVE

Toxicants may act directly on the genetic material of living organisms by inducing mutations. At ERL-N, Chinese hamster lung fibroblast tissue culture cells (V79) are routinely used to evaluate mutagenic effects of single compounds and complex mixtures, including marine sediment extracts and their fractions. These cells have 21 ± 2 large chromosomes, a rapid growth rate (doubling time of 12 hours) and a high plating efficiency. This tissue culture system is commonly used for a variety of short-term genetic assays, one of which is the determination of genetic damage and mutational events by cytogenetic analysis (direct observation of chromosomes, the genetic material) of sister chromatid exchanges (SCEs). A sister chromatid exchange represents the breakage and reciprocal exchange of identical DNA material between the two sister chromatids of a chromosome. These methods have been used to evaluate effects of single compounds such as mitomycin C, benz(a)pyrene and to whole sediment extracts and sediment fractions from Black Rock Harbor, CT, central Long Island Sound, NY, Allen Harbor, RI and Narragansett Bay, RI.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Subculturing.

See SOP on subculturing for materials and equipment.

B. Plating Preparations. Note: Manipulation of cells require aseptic techniques, which include appropriate clothing, flow hoods and decontamination procedures.

- Sterile plastic tissue culture plates.
 - 60 x 15mm NUNC (Daigger Scientific 150-288) for toxicity testing.
 - 100 x 15mm NUNC (Daigger Scientific 150-350) for subculturing and for plating and cell exposure.
- Sterile 5 3/4" Pasteur pipettes (Daigger Scientific 7760-A12).
- 15 ml sterile Corning centrifuge tubes, capped (Daigger Scientific 25310-15) for serial dilutions of V79 cells for toxicity testing.

C. Plating Procedure (additional materials and equipment).

- Hemocytometer for determining the concentration of the cell suspension.

D. Dosing (additional materials and equipment). Note: Use of carcinogens, mutagens, teratogens, toxic chemicals and complex mixtures require appropriate protective clothing and engineering controls.

- Biohazard hood (Baker Co. BC-6) to protect researchers from exposure to toxicant chemicals and sediment extracts, and to maintain a sterile environment for V79 tissue culture cells.
- 50 ml sterile Corning centrifuge tubes, capped (Daigger Scientific 25339-50) for preparation of "dosed" media.
- 250 ml Erlenmeyer flasks (Daigger Scientific 25600), for the preparation of dosing media, with and without S9 activation.
- Toxic waste disposal.
 - Ziplock waste bags (Arnold David Cohen 12"x15" 6 mil) for disposal of solid toxic waste.
 - Cubitainers for disposal of liquid toxic waste.
- S9 rat liver homogenate (Organon Technika 36431) for exogenous metabolism.
- β -Nicotinamide Adenine Dinucleotide Phosphate (NADP/Sigma N0505) and D-Glucose 6-Phosphate (G6P/Sigma G7250) used with S9 in activation system.

E. Media Change (additional materials and equipment).

- Bromodeoxyuridine (BRDU/Sigma B5002), a thymidine analog needed for differential staining. Note: BRDU is a carcinogen.

F. Harvest (additional materials and equipment).

- Potassium Chloride for preparation of a hypotonic solution.
- Acetic Acid (Daigger Scientific C005-Q38) to fix cells.
- 100% Ethanol to fix cells.
- 15 ml nonsterile Corning centrifuge tubes for containing cell suspension.
- Colcemid (Gibco 120-5211) to inhibit mitosis at metaphase.
- Centrifuge to concentrate cells.
- Nonsterile 5 3/4" Pasteur pipettes (Daigger Scientific 7760-A12) for reagent and cell manipulation.

G. Slide Preparation (additional materials and equipment).

- Slide warmer to heating slides.
- Corning glass slides (Daigger Scientific 6686-Q18).
- Magnetic stirring plate and magnetic bars for the preparation of reagents.

- Ultraviolet light source consisting of a Spectral lamp power supply (Ealing Optical Div 26-2683), Mercury (high press.) spectral lamp (Ealing Optical Div 26-2865), and a Spectral lamp house (Ealing Optical Div 27-1254), for fluorescent staining.
- 0.1M Citric Acid for buffer.
- 0.2M Dibasic Sodium Phosphate (Na_2HPO_4) for buffer.
- Hoescht 33258 (Crescent Chemical Co. 33217).
- Giemsa (Daigger Scientific C363-H12), for staining.
- Staining equipment (staining dishes and racks).
- Corning #2935 Coverslips (Daigger Scientific 6669-F81).
- Mounting media (Daigger Scientific 6705-A10) for applying coverslips.

H. Concurrent Toxicity Test "Take down" (additional materials and equipment).

- 70% ETOH to fix cells.
- Crystal violet stain to facilitate visualization of colonies for counting.

I. Scoring Procedure (additional materials and equipment).

- Zeiss research microscope
- Immersion oil (Daigger Scientific C409-L78)
- 15x oil objective (RAININ Instr 461525) to scan slides.
- 63x oil objective (RAININ Instr 46-18-40) for scoring SCEs.

III. METHODS

A. Subculturing. Note: Manipulation of cells requires aseptic techniques.

6-8 plates are used during a typical SCE test consisting of a blank or control, positive control, solvent control and three to four toxicant doses, all with and without S9 activation. See subculturing SOP for detailed description of procedures.

B. Plating Preparations.

Media is prepared simultaneously for plating, dosing and renewal. Earles Balanced Salts Solution containing 5% fetal bovine serum (5% Earles) is prepared for the plating procedure. Three small plates (60x15mm NUNC sterile plastic tissue culture plates) are required for each treatment of the toxicity test, and two to three large plates (100x15mm NUNC sterile plastic tissue culture plates) are needed for the SCE test.

1. Media Preparations (for plating, dosing and media change procedures)

Equal amounts of media, for plating, dosing, and media change procedures, are prepared. Use the following equation to find the total amount of media to prepare for each procedure:

$$\begin{array}{l} \text{media for} \\ \text{toxicity test} \end{array} = (\text{no. doses})(\text{no. plates/dose})(4\text{ml media/plate}) \quad (1)$$

**ERL-N STANDARD OPERATING PROCEDURE
V79/SISTER CHROMATID EXCHANGE ASSAY**

ERL-N SOP 1.03.012
REVISION 0
DECEMBER 1990
PAGE 4 OF 12

media for SCE test = (no. doses)(no. plates/dose)(10ml media/plate) (2)

tot. media = (media for toxicity test) + (media for SCE test) (3)

Equations (1) and (2) are used to subdivide media during plating and media change procedures.

See SOP on Subculturing for media preparation with the following exceptions:

	Plating	Dosing	Media Change
Add	0.5% Penn-Strep	0.5% Penn-Strep	0.5% Penn-Strep
change	5% fetal bovine serum	no serum	5% fetal bovine serum

2. 5% Earles

Earles balanced salts solution containing 5% fetal bovine serum (5% Earles) is needed to inhibit trypsin during the plating procedure, and for cell dilutions for toxicity plating. Use the following equation to calculate the total amount of 5% Earles:

5% Earles = 27 ml + (3 ml) (no. plates trypsinized))

9 ml of 5% Earles is pipetted into each of three sterile, capped 15 ml centrifuge tubes for toxicity test dilutions, tubes are labelled (A) (B) and (C). The remaining 5% Earles is used to prepare the cell suspension.

3. Labelling

Plates are labelled according to their concentration. Each plate is given an appropriate toxicant code followed by the appropriate S9 activation code. Table of codes is as follows,

Toxicant: BL, for blank dose,
(a chemistry prepared blank for sediment extracts.)
PC, for the positive controls,
(mitomycin C.)
CT, for controls,
(Contains solvent equal to that of the highest dose.)
A, highest concentration.

Z, lowest dose.

S9 activation: +, contains cofactors for S9 activation.
-, lacks the cofactors for S9 activation.

C. Plating.

1. Preparations.

- a. Divide media for toxicity plates and SCE plates, using the values obtained from equations (1) and (2).
- b. Use an inverted microscope to examine cultures for contamination and growth.

2. Cell suspension.

- a. Rinse 6-8 V79 cell cultures twice with 5 ml of Earles Balanced Salts Solution and vacuum.
- b. Add 0.5 ml trypsin to each plate and allow to stand for 5 min or until cells begin to detach.
- c. Inhibit trypsin by pipetting 3 ml of 5% Earles onto each plate.
- d. Remove cells with vigorous pipetting (repeatedly drawing cell suspension into the pipette and dispensing it back onto the plate to loosen the other cells), then add cell suspension back to the bottle of 5% Earles.

3. Toxicity test dilutions.

- a. Mix the cell suspension and add 1 ml of cell suspension to the first dilution of 1:10 (A), mix A and pipette 1 ml of A into second dilution of 1:10 (B), mix B and pipette 1 ml of B into the third dilution of 1:10 (C).

4. Hemocytometer counts of cell suspension (see SOP).

- a. Mix the cell suspension from C.2.
- b. Using a sterile 5 3/4" pipette, remove a sample of cell suspension to load one side of a hemocytometer, discard the pipette. Repeat to load the other side of the hemocytometer.
- c. The number of cells within the center grid on each side is counted and

averaged. The number of cells per ml in the cell suspension is as follows:

$$\text{cells per ml} = \text{average} \times 10^4$$

5. Toxicity plating.

a. Each small plate (a 60x15mm sterile NUNC tissue culture plate) receives 100 cells (25 cells/ml). The volume of the toxicity test cell dilution C to add to the total volume of toxicity media is calculated as follows, (cells per ml as calculated in section C.4.c.)

$$\text{dilution factor (DF)} = \text{cells per ml}/25$$

All toxicity dilutions are 1:10, therefore:

dilution (A) contains $\text{DF} \times 10^3$ cells

dilution (B) contains $\text{DF} \times 10^2$ cells

dilution (C) contains $\text{DF} \times 10$ cells

$$\text{vol C} = (\text{vol tox media})/(\text{DF} \times 10)$$

b. Mix toxicity media with cell suspension C and add 4 ml to each plate.

c. Return plates to the incubator for 24 hours to allow for cell attachment.

6. SCE plating

Each SCE plate (a 100x15mm sterile NUNC tissue culture plate) receives 11×10^5 cells. The volume of cell suspension, from section C.2., to add to the total volume of media is calculated as follows, (cells per ml as calculated in section C.4.c.)

$$\text{dilution factor} = \text{cells per ml}/11 \times 10^4$$

$$\text{vol CS} = \text{vol SCE media}/\text{DF}$$

a. Mix SCE media with cell suspension, and add 10 ml to each plate.

b. Return plates to the incubator for 24 hours to allow for cell attachment.

D. Dosing. Note: All procedures are conducted using aseptic techniques and to protect the researcher from potential toxicants.

1. Media.

a. With cofactors.

1. In a 250 ml Erlenmeyer flask add thawed S9 to half of the media (final concentration of 0.789 mg/ml protein). Note: Protein content of S9 batch is determined by the manufacturer.

2. Add cofactors (final concentrations of 0.63 mg/ml NADP and 0.27 mg/ml G6P).

b. Without cofactors.

1. In a 250 ml Erlenmeyer flask add thawed S9 (as calculated above) to the remaining half of the media.

2. Solvents.

Solvents are used in the following order of preference: water, Dimethylsulfoxide, and alcohol. The percentage of solvent used per plate is not to exceed 5%. Unless historical literature is available, the use of solvents requires both toxicity and SCE testing to determine their cytotoxic and genotoxic potential. All plates are equilibrated to contain solvent equal to that found in the highest dose.

3. Dosing.

a. Pipette media into prelabeled 50 ml centrifuge tubes.

b. Add solvent and test chemical or mixture

c. Vacuum media from all plates.

d. Beginning with the blank and proceeding from the lowest to the highest dose add media to cells. Toxicity plates receive minimum of 3 ml of dosed media and up to 4 ml. SCE plates can receive a minimum of 7 ml and up to 10 ml. Record the time.

e. Return plates to the incubator for 5 hours.

E. Media Change

1. Divide media for renewal (referred to as "clean media") using volumes calculated by equation (1) and (2).

2. Vacuum off exposure media from the toxicity plates and rinse with 4 ml of Earles balanced salts solution.

3. Vacuum plates and add 4 ml of clean media to each plate.

4. Return plates to the incubator for 6 days.

5. Vacuum off exposure media from plates, rinse with 7 ml of Earles balanced salts solution.
6. Vacuum off Earles balanced salts solution and add 10 ml of media containing 1×10^{-5} g/ml of BRDU. Note: BRDU is a carcinogen.
7. Return plates to the incubator for 20 hours.

F. Harvest

1. 1 1/2 hours prior to the harvest, add 0.5 ml of colcemid (one vial reconstituted with 10 ml of sterile DI) to each SCE plate to stop the cell cycle at metaphase, and return SCE plates to the incubator.

Reagent and materials preparations:

- a. KCl hypotonic solution (5.49 ml of KCl to 1 liter DI).
- b. Carnoy's fixative (1 part acetic acid : 3 parts ethanol).
- c. 15 ml noncapped, unsterile centrifuge tubes (labelled according to dose)
- d. 5% Earles Balanced Salts Solution, to stop trypsin. (As calculated in media preparations).

2. Harvest

This involves the harvesting of the cells from the SCE plates. Cells are then placed in a KCl hypotonic solution to expand the cytoplasm and spread the metaphase chromosomes. Cells are fixed three times with Carnoy's, and stored overnight prior to slidemaking.

- a. Vacuum plates, rinse with 5 ml of Earles balanced salts solution, repeat and vacuum off final rinse.
- b. Add 0.5 ml of trypsin for 5 min and stop trypsin with 5 ml of 5% Earles.
- c. Vigorously pipette cells off plate and put cell suspension into corresponding 15 ml centrifuge tube.
- d. Centrifuge cell suspensions (all cell centrifugations are at the same speed) for 5 minutes at 800 rpm, discard supernatant.
- e. Add 4 ml of KCl hypotonic solution, resuspend and let stand for 10min.
- f. Centrifuge and discard supernatant.

- g. Fix with 4 ml Carnoy's fixative, resuspend cells and let stand for 10min.
- h. Centrifuge cell suspension and discard supernatant.
- i. Repeat fix step.
- j. Add 4 ml of Carnoy's fixative, resuspend, cover with aluminum foil or parafilm, and refrigerate overnight.

G. Slide Preparation

1. Slide making

- a. Centrifuge, discard supernatant to about 0.5 ml cell suspension and fix, resuspend using a nonsterile 5 3/4" pasteur pipette.
- b. Drop 2 to 3 drops of cell suspension on one end of a microscope slide, blow down the length of the slide, and place slide immediately on a slide warmer to dry. Slides are labelled 'V' (indicating V79 cells) followed by consecutive numbering, for blind scoring.

2. Slide staining

- a. Stain slides in Hoechst stain (45 µg/ml McIlvaine's buffer pH 7) for 10 min, and rinse in DI.
- b. Submerge slide in McIlvaine's buffer (pH 8) under ultraviolet light for 60 min, and rinse in DI. Note: Precautions are necessary to protect eyes and skin from exposure to ultraviolet light.
- c. Stain in Giemsa (1ml/25ml deionized water) for 9 min, rinse in DI, airdry, and coverslip. Note: Giemsa stain is a poison.

H. Concurrent Toxicity Test "Take down"

Seven days after initiation, the 100 cells seeded have multiplied to form visible colonies.
Note: Fixing, staining and counting of colonies do not require sterile techniques.

- 1. Pour off media and add enough 70% ethanol to cover the bottom of the plate for 10-15 min.
- 2. Pour off the 70% ethanol and replace with crystal violet stain to cover bottom of plates for 10-15 min.
- 3. Rinse plates in warm water and let airdry.

I. Scoring Procedures

1. Concurrent toxicity test scoring

- a. The colonies are counted, either by hand or using an image analyzer, and recorded.

2. SCE test scoring

- a. Slides are scanned, using a 16x oil objective, for suitable chromosome spreads (A "spread" is a group of chromosomes, usually of the same cell). For each slide, counts of the number of chromosomes and the number of SCEs per spread are recorded. Other observations (chromosomal aberrations, slide quality, etc.) are also recorded.

IV. STATISTICAL ANALYSIS AND DATA USAGE.

A. Toxicity tests

1. The mean colony number per dose is used to find the percent survival of a dose as compared to the control or blank for that test.

B. SCE tests.

1. An "SCEFACT" (no. SCEs / no. Chromosomes) and a "LOGRESUL" ($\log(\text{SCEFACT} + 1)$) is calculated for each spread scored.
2. The mean and standard deviation of the SCEFACT and LOGRESUL is calculated for each plate.
3. The mean and standard deviation, of the mean SCEFACT and mean LOGRESUL for each plate, is calculated to find the mean for the dose.
4. Corresponding graphs of the data are then used to illustrate the results.
5. The mean SCEFACT for each dose is normalized to the blank or control. Doubling over controls is considered a significant response.

V. TROUBLE SHOOTING

A. Contamination

1. The test should be monitored daily for fungal or bacterial contamination. Any contaminated plates should be removed, taped with autoclave masking tape, wrapped

in foil and autoclaved.

2. Check incubator for contamination before and after each test is completed.
3. Clean incubator before putting a new test into the incubator. Note: See SOP on Clean Room Maintenance.

B. Slide Quality

1. Prepare extra slides to pre-test stains and buffers.

- a. Chromosome quality (colcemid related problems)

Long, stringy chromosomes indicate that the colcemid was not left on long enough. If the chromosomes appear short and fat leave colcemid on for a shorter time. Adjust time by adding colcemid earlier or later than 1 1/2 hours.

- b. Staining qualities

1. BrdU related

Dark stained chromatids indicate that the BrdU was not on long enough. If light chromatids appear, then the BrdU was on too long. Adjust by conducting the harvest earlier or later as needed.

2. McIlvaines (pH buffer) related

This buffer is used at a pH of 7 for the Hoescht stain and at a pH of 8 for use under the UV light source. If the chromosomes appear bloated and balloon-like, then prepare a new batch of buffer and stain, then repeat the entire staining process on a single extra slide.

3. Giemsa related

If the Giemsa is old and at the bottom of the bottle, then it is important to check the quality of the stain by staining a single slide. If chromosomes do not stain well, then prepare a new batch of stain with a new bottle of Giemsa.

- c. Spread quality.

1. KCl hypotonic related

If the chromosomes spreads appear clumped or cytoplasm still exists, then there is a problem with the hypotonic solution.

2. Carnoy's fix related

If the chromosomes are scattered around slide, and everything else appears normal, the resuspension stage of fixation was too brutal and broke up the spreads. Therefore, be more gentle during fixation.

C. Scoring

1. Scorers

Scoring of concurrent toxicity tests and SCE tests should be done by one person to prevent possible variabilities between scorers within a test.

2. Recordkeeping

Observations are recorded on data sheets and transferred to the computer for statistical purposes.

3. SCE scoring

Only those spreads and chromosome which are readable are scored to eliminate false readings.

VI. REFERENCES

None.

ERL-N STANDARD OPERATING PROCEDURE
GROWTH AND SCOPE FOR GROWTH MEASUREMENTS
WITH *Mytilus edulis*

ERL-N SOP 1.03.013
REVISION 0
FEBRUARY 1991
PAGE 1 OF 29

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I. OBJECTIVE

This standard operating procedure describes the methods to determine growth and the scope for growth (SFG) index using the blue mussel, *Mytilus edulis*.

The SFG index (Warren and Davis, 1967) is a measure of the energy available to an organism for somatic and reproductive growth after accounting for routine metabolic costs. Determination of the SFG index requires the measurement of three physiological parameters: clearance rate, respiration rate and food assimilation efficiency. Clearance rate and assimilation efficiency measurements are used to determine total amount of energy available, while respiration rate is used to estimate metabolic energy costs.

Applications of the SFG index with *Mytilus edulis* range from ration effects (Thompson and Bayne, 1974) to estuarine pollution gradient effects (Widdows, *et al.*, 1981). Bayne *et al.* (1981) found reduced growth and ultimately reduced fecundity and fitness in *M. edulis* after sustained reduction in SFG. At ERL-N, experiments are conducted to test for relative differences in SFG among field stations. Mussels of similar physiological condition should demonstrate similar SFG responses under standardized conditions; therefore, differences in SFG are attributed to persistent physiological effects of field exposure. However, comparisons among separate experiments should not be made on absolute SFG values due to seasonal changes in mussel physiology.

II. MATERIALS AND EQUIPMENT

A. Mussel Collections and Deployments

- 1/2" polyethylene marine netting (ADPI Enterprises, Inc.), 1 ft², to make mussel baskets
- Hospital identification bracelet (Big Band series 140, adult)
- Lashing ties (Ty-Rap)

ERL-N STANDARD OPERATING PROCEDURE
GROWTH AND SCOPE FOR GROWTH MEASUREMENTS
WITH *Mytilus edulis*

ERL-N SOP 1.03.013
REVISION 0
FEBRUARY 1991
PAGE 2 OF 29

B. Algal Culture

- Constant temperature culture room held at 21°C and light intensity of 75-100 $\mu\text{E m}^{-2} \text{sec}^{-1}$
- Autoclave
- Sterile 5 and 1 ml pipettes
- Culture bottles, 500 ml
- Carboys, 18 L
- Foam plugs, 1.5 cm and 5 cm in diameter
- De-ionized water
- Natural sea water
- Charcoal water filter
- Cartridge filters, 15 and 1 μm
- Modified (Spotte et al., 1984) GP2 artificial sea water (SAIC, 1990):
 - Sodium chloride (NaCl)
 - Sodium sulfate (Na_2SO_4)
 - Potassium chloride (KCl)
 - Potassium bromide (KBr)
 - Sodium borate ($\text{Na}_2\text{B}_4\text{O}_7$)
 - Magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)
 - Calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)
 - Strontium chloride ($\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$)
 - Autoclavable bottle, 10 L
 - Top loading balance (with ± 1.0 g precision)
 - Analytical balance (with ± 0.1 g precision)
- 4X Provasoli's Enriched Seawater (PES) nutrient media (McLachlan, 1973):
 - Sodium EDTA (Na_2EDTA)
 - Volumetric flask, 2 L
 - Ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$)
 - Manganous sulfate (MnSO_4)
 - Zinc sulfate (ZnSO_4)
 - Cobalt sulfate (CoSO_4)
 - Sodium nitrate (NaNO_3)
 - Boric acid (H_3BO_3)
 - Sodium phosphate ($\text{Na}_2\text{H}_2\text{PO}_4$)
 - Hydrochloric acid (HCL)
 - Thiamine
 - Biotin
 - Cyanocobalamine (B12)
 - Volumetric flask, 250 ml
 - Autoclavable screw-top test tubes, 20 ml
 - Disposable pipettes
 - Autoclavable jug for media storage
 - Small and large weigh boats
 - Spatula

ERL-N STANDARD OPERATING PROCEDURE
GROWTH AND SCOPE FOR GROWTH MEASUREMENTS
WITH *Mytilus edulis*

ERL-N SOP 1.03.013
REVISION 0
FEBRUARY 1991
PAGE 3 OF 29

- Magnetic-stirrer hot plate
- Analytical balance (with ± 0.1 g precision)
- Microbalance (with ± 0.001 g precision)

C. Clearance Rate Determination

- 40 Nalgene square bottles, 1 L
- T-connectors, 15 mm
- Aspirator bottle, 4 L
- Peristaltic pump
- Cartridge water filters, 15 and 1 μ m
- Natural sea water
- Isochrysis galbana (clone T-Iso)
- Nucleopore polycarbonate filters, 1 mm
- Vacuum pump
- Collection vials
- Coulter Counter (Model ZM or Model TAPI)

D. Respiration Rate Determination

- Flow-through water bath
- Glass respirometer vessel, 430 ml
- Air-driven magnetic stirrer
- Stir-bar
- PO₂ electrode (Radiometer E5047)
- PO₂ electrolyte solution
- PO₂ zeroing solution
- Accessory kit for PO₂ electrodes
- Oxygen meter (Radiometer Model PHM71)
- Strip chart recorder
- Bristle brush

E. Assimilation Efficiency and Mussel Dry Weight

- Pre-weighed 1 cm² aluminum pans
- Aluminum dishes
- Muffle furnace
- Microbalance (Cahn model 30)
- Disposable Pasteur pipets
- Suction filter apparatus
- Rotary valve vacuum pump
- Nucleopore polycarbonate filters, 0.45 μ m
- Isotonic ammonium formate
- Watch glass
- Plastic spatula
- Volumetric flask, 2 L

ERL-N STANDARD OPERATING PROCEDURE
GROWTH AND SCOPE FOR GROWTH MEASUREMENTS
WITH *Mytilus edulis*

ERL-N SOP 1.03.013
REVISION 0
FEBRUARY 1991
PAGE 4 OF 29

- Drying oven
- Top-loading balance
- Shucking knife
- Vernier caliper
- Forceps

III. METHODS

A. Mussel Collections and Deployments

1. Blue mussels, *Mytilus edulis*, are collected from locations previously determined to be "clean" with respect to contaminants (eg. lower Narragansett Bay, RI, and East Sandwich, MA). After collection, mussels are returned to the laboratory and maintained in ambient unfiltered Narragansett Bay sea water until deployment. Mussels are collected within 2-4 days of deployment to minimize laboratory holding effects. See Benthic Organism Collection SOP for collection method and equipment.
2. Organisms are sorted to obtain a uniform size range (usually 5-6 cm) to minimize effects due to size differences.
3. A subset of mussels are sorted further to obtain a narrower size range (± 1 mm) for shell growth determinations. A total of 15 marked mussels are placed in each replicate basket.
 - a. The mussels are individually numbered with a diamond engraving bit. Because this is a non-destructive process, these mussels can also be used for additional chemical analyses or distributed to other researchers for additional testing.
 - b. The anterior-posterior shell length is measured to the nearest 0.1 mm with a vernier caliper. Ten percent of the mussels are re-measured to check the precision of the measuring technique.
4. The number of replicates per station and the number of stations included in a study is contingent on the experimental design. In typical studies conducted at ERL-N, four replicates at each station are sufficient. Each replicate consists of a small polyethylene basket (obtained commercially or fabricated in-house) containing 25 mussels (enough for both chemical analyses and biological measurements).
5. Field deployments can be accomplished using either diver- or surface-tended stations. The advantage of diver-tended stations is that the potential for vandalism is reduced. However, this approach is more costly and time consuming, and can be a health hazard if the study site is severely contaminated. A second approach is to use a sub-surface buoy system which is attached to a surface float. Each deployment consists of a series of replicate moorings (such as a concrete block) connected together in a line (Fig. 1). See the Caged Bivalve Deployment SOP for deployment methods and equipment.

**ERL-N STANDARD OPERATING PROCEDURE
GROWTH AND SCOPE FOR GROWTH MEASUREMENTS
WITH *Mytilus edulis***

ERL-N SOP 1.03.013
REVISION 0
FEBRUARY 1991
PAGE 5 OF 29

B. Algal Culture

Standardized laboratory measurements of SFG requires a suitable unicellular food source. The algal species utilized at ERL-N is a strain of Isochrysis galbana, clone T-Iso. PES nutrient media has been found to provide the most reliable algal growth of T-Iso.

1. Media

The following stock solutions are prepared and ultimately are added to the PES media:

a. FeCl solution:

1. FeCl is prepared by dissolving approximately 100 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 100 ml HCl. This is a molar:molar (Fe:Cl) solution. To ensure a 1:1 molar ratio, measure the 100ml HCL, and in it dissolve just enough $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. If too much $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ is added, add HCl drop by drop until the $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ is completely dissolved. This procedure should be completed in a hood, with proper safety attire.

2. Add this 100 ml FeCL solution to approximately 800 ml DI.

3. Then add 0.66 g Na_2EDTA and bring to the final volume of 1 L.

**** The FeCl solution must be refrigerated.**

b. P11 Metals stock solutions (each stock is made in a separate 100 ml volumetric flask and stored in an individual acid stripped bottle):

1. Add 5.0 g Na_2EDTA to 100 ml DI.

2. Add 0.49 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ to 100 ml DI.

3. Add 1.64 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ to 100 ml DI.

4. Add 0.22 g $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ to 100 ml DI.

5. Add 0.048 g $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ to 100 ml DI.

c. 4X Vitamin solution:

1. Into a 250 ml volumetric flask add:

- 1.0 g Thiamine
- 10.0 mg Biotin
- 20.0 mg B12

2. Vitamins are stored in the refrigerator. Low heat and stirring are recommended as the vitamins are difficult to dissolve. Biotin and B12 should

ERI-N STANDARD OPERATING PROCEDURE
GROWTH AND SCOPE FOR GROWTH MEASUREMENTS
WITH *Mytilus edulis*

ERI-N SOP 1.03.013
REVISION 0
FEBRUARY 1991
PAGE 6 OF 29

be weighed on a microbalance accurate to 1.0 μg .

3. After the vitamin solution is prepared, 10 ml aliquots are added to loosely-stoppered, autoclavable, screw-top test-tubes and autoclaved at 250°C for 1 min. The tubes are then cooled to room temperature and their screw-tops tightened. The tubes are stored at 4°C.

d. 4X PES nutrient media:

1. Fe-EDTA solution:

a. Na_2EDTA is dissolved in DI and FeCL solution over heat for about 1 hr.

b. Into a 2 L volumetric flask, add:

- 200 ml FeCL solution (as prepared above)
- Approximately 600 ml DI
- 1.32 g Na_2EDTA

2. P11 metal solution:

a. Into a 250 ml volumetric flask add the following amounts of the metal stock solutions (as prepared above), adding the Na_2EDTA solution first:

- 40 ml Na_2EDTA
- 20 ml $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$
- 20 ml MnSO_4
- 20 ml ZnSO_4
- 20 ml CoSO_4

b. Mix and add 130 ml DI.

3. Add to the Fe-EDTA solution (when the Fe-EDTA solution is completely dissolved):

- 28.0 g NaNO_3
- 4.56 g H_3BO_3
- 1.72 g $\text{Na}_2\text{H}_2\text{PO}_4$

Rinse the neck of the volumetric flask with DI after each chemical addition and ensure that each chemical is completely dissolved before adding the next one.

4. The trace metal solution is added and mixed, along with 3.2 ml potassium iodide, and the solution brought to its final volume of 2 L with DI.

5. The solution is poured into a bottle and autoclaved for 10 minutes at 250°C on liquids cycle.

6. Allow the solution to cool to room temperature.

7. Two 10 ml test tubes of 4X vitamins are warmed to room temperature and added to the PES media (one 10 ml test tube of 4X vitamin solution is required for each liter of media). The mixture is shaken well and stored in the dark at room temperature.

e. GP2 artificial seawater:

The original recipe of Spotte *et al* (1984) prepared an artificial sea water solution at 35 ppt. The recipe was modified to resemble natural sea water in Narragansett Bay, which has a salinity of 30 ppt.

1. The following recipe is prepared in a 10 L autoclavable bottle. Each chemical is completely dissolved prior to addition of the following chemical.

- 210.30 g NaCl
- 35.20 g Na_2SO_4
- 6.10 g KCl
- 0.88 g KBr
- 0.34 g $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$
- 95.00 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
- 13.20 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
- 0.20 g $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$
- 1.70 g NaHCO_3

Bring to final volume of 10 L.

2. Algal Cultures

All cultures of T-Iso are maintained in a culture room under constant temperature (21°C) and light (75-100 $\mu\text{E m}^{-2} \text{sec}^{-1}$) conditions. Sterile materials are used to keep the cultures free from contamination. It is important that cultures be checked routinely for contaminating bacteria or other unicellular algal species.

a. Primary and Secondary Stock Cultures

Primary stock cultures are maintained under aseptic, mono-clonal conditions because SFG calculations are based on the food value of pure algae. The primary stock culture is always maintained separately, and is used only for inoculation of secondary culture on a routine basis or if the secondary culture becomes contaminated. Procedures for primary and secondary culture maintenance are otherwise similar.

1. Natural sea water for primary and secondary stock cultures is filtered through both a charcoal cartridge filter and a 1 μm cartridge filter into acid stripped 500 ml bottles.

**ERL-N STANDARD OPERATING PROCEDURE
GROWTH AND SCOPE FOR GROWTH MEASUREMENTS
WITH *Mytilus edulis***

ERL-N SOP 1.03.013
REVISION 0
FEBRUARY 1991
PAGE 8 OF 29

2. The bottles are filled with a 50-50 mix of natural sea water and sterilized GP2 artificial sea water, and autoclaved at 240°C for 30 minutes. The tops of bottles are either wrapped with aluminum foil, or loosely capped to avoid contamination before use.
3. Secondary cultures are inoculated by adding about 5 ml of primary culture to a sterilized bottle containing 500 ml of the natural/artificial sea water mixture.
4. Approximately 1.5 ml of 4X PES algal nutrient media is added to each culture to promote rapid growth.
5. Cultures are aerated by placing a 1 ml pipette in the bottle and connecting it to a compressed air system in the algae culture room. Sterilized foam plugs are placed in the neck of the bottle to avoid contamination.
6. Secondary cultures require about 5 to 7 days to reach the density required to successfully inoculate working algal stocks used to feed mussels.

b. Working Stock Cultures

The working algal stock is cultured in 18 L carboys (e.g. polycarbonate spring-water bottles). Approximately 76 L of algae are required to conduct SFG measurements on 36 mussels. Generally, 5-6 carboys are maintained at all times. Again, it is essential that these cultures remain pure because the SFG calculations are based on the food value of pure algae.

1. Each carboy is filled with 1 μm filtered natural sea water and autoclaved for 30 minutes at 240°C.
2. Fifty ml of 4X PES nutrient media and 500 ml of secondary stock culture are added to each 18 L carboy to inoculate the carboy.
3. The top of a sterile 5 ml pipette is pushed through a large (30 mm diameter) sterile foam plug, inserted into the carboy and connected to a compressed air supply.
4. The density of algae in the carboy should reach approximately 1×10^6 cell ml^{-1} after about 5 to 7 days of growth.

C. Clearance Rate Determinations

Clearance rate is defined as the volume of water completely cleared of particles $>2 \mu\text{m}$ in a per unit of time (Widdows *et al.*, 1979).

1. Distribution system preparation.

- a. Sand-filtered (10 μm) ambient sea water available in the laboratory is pumped

through 15 μm and 1 μm cartridge filters, to a 2 ft diameter aerated head tank (Fig. 2).

b. Filtered sea water is gravity-fed from the head tank into a mixing chamber along with T-Iso added via peristaltic pump. The mixing chamber is tightly stoppered which creates a closed system, and causes the rate of inflow to equal the rate of outflow. If only one distribution system is in use, the flow is lower ($\sim 850 \text{ ml min}^{-1}$) than if four distribution systems are in use (3.5 L min^{-1}). The pump is adjusted to provide a final algae concentration of approximately $20,000 \text{ cells ml}^{-1}$.

c. The mixture is then gravity-fed to a distribution apparatus fitted with ten 2.0 mm capillary tubes and an overflow tube (Fig. 2). Four distribution apparatuses are needed to efficiently measure clearance rates. Each capillary tube is adjusted to length to allow a flow rate of 4.5 L hr^{-1} . Clearance rate chambers consist of 1 L Nalgene bottles fitted with overflow t-connectors (Fig. 3).

2. Organism preparation.

a. Two mussels are randomly selected from each basket, placed into individual clearance rate chambers and allowed to feed overnight.

3. Coulter Counter calibration (model TAPI).

The Coulter Counter should be calibrated whenever the aperture tube is changed, before each set of critical samples, and after any movement, repair, or adjustment of the instrument.

a. An aperture tube with a range appropriate for the sample material (i.e. 2% to 40% of the aperture diameter) is selected and cleaned with 7X detergent and DI rinse.

b. 500 ml of electrolyte, (i.e. seawater) is filtered through a 0.2 μm filter at least 2 times.

c. The aperture tube is then rinsed and filled $\frac{1}{2}$ way with electrolyte.

d. The reservoir is then filled with approximately 200 ml of electrolyte.

e. Three clean 25 ml scintillation vials are filled with 10 ml each with the filtered sea water.

f. Turn the power on and allow the instrument and pump to warm up for at least 30 min.

g. To obtain a background count on filtered sea water:

1. Use the 0.5 ml setting for the 100 μm aperture tube.

2. Set DISPLAY to DIF.

**ERL-N STANDARD OPERATING PROCEDURE
GROWTH AND SCOPE FOR GROWTH MEASUREMENTS
WITH *Mytilus edulis***

ERL-N SOP 1.03.013
REVISION 0
FEBRUARY 1991
PAGE 10 OF 29

3. Set READING to TOTAL COUNT.
 4. Set SAMPLING to MANOMETER.
 5. Set ACTIVE CHANNEL to "15-2".
 6. Set APERTURE CURRENT to POS or NEG.
 7. Purge the aperture by opening the sample valve followed by the flushing valve. To stop, close the flushing valve first, then the sample valve.
 8. Open the sample valve and allow the instrument to draw a sample. The total count should be less than 650. If it is greater than 650, re-filter the sea water.
- h. To prepare a standard bead count:
1. Select the appropriate bead size (i.e. 4-10% of the aperture diameter) and shake well.
 2. Add 1 drop of bead solution to 100 ml filtered seawater. Transfer 10 ml to a clean 25 ml vial and label "stock".
 3. Sonicate for 1 minute.
- i. To obtain a bead count:
1. Set SIZE CAL CHANNEL to appropriate channels where most beads will be counted.
 2. The total count should be between 7000 to 20,000 for the 100 μ m aperture. If not, either dilute or add more beads to the stock (re-sonicate if more beads are added).
- j. To adjust the size calibration control:
1. Set the meter switch to CAL position.
 2. Record SIZE CALIBRATION, SIZE CAL CHANNEL, and APERTURE MATCHING SWITCH settings on worksheet (Table 1).
 3. Open sampling valve, and allow the instrument to reset.
 4. Rotate SIZE CALIBRATION control until the meter reads center scale.
- k. Determine the new SIZE CALIBRATION (A_2) setting through the following formula:

$$A_2 = A_1 \times (D_1/D_2)^2$$

**ERL-N STANDARD OPERATING PROCEDURE
GROWTH AND SCOPE FOR GROWTH MEASUREMENTS
WITH *Mytilus edulis***

ERL-N SOP 1.03.013
REVISION 0
FEBRUARY 1991
PAGE 11 OF 29

where: A_1 = old SIZE CALIBRATION setting, above
 A_2 = new SIZE CALIBRATION setting
 D_1 = nominal diameter of beads
 D_2 = nearest particle diameter shown on worksheet (Table 1)
 W_1 = upper (larger) channel where particles accumulated
 W_2 = channel number corresponding to D_2 , on worksheet from previous calibration with this aperture diameter. (W_1 will usually be the same as W_2).

1. Record the channel number where the principal accumulation now occurs, as well as the new calibration setting onto the worksheet. Also record the serial no. of the aperture tube.

2. Fill in the remaining channel numbers, from 1 to 16. Channels 2 and 15 should be at the lower and upper limits of the aperture range, respectively (i.e. 2% and 40 % of the aperture diameter).

3. If channel 2 falls below the desired range, multiply the SIZE CALIBRATION setting by 2 and record on the worksheet.

4. If channel 15 falls above the desired range, divide the SIZE CALIBRATION setting by 2 and record onto the worksheet.

4. Coulter Counter operation.

It is usually convenient to prepare the Coulter Counter for particle measurements during the period of mussel acclimation.

a. First, approximately 500 ml of 1 μ m filtered sea water is added into the reservoir.

b. The aperture is purged by first opening the sample valve followed by the flushing valve.

c. To stop, close the flushing valve first, then the sample valve. Care must be taken to ensure this step is performed in the proper sequence as improper procedures may result in allowing sea water to flow into the mercury column, making the Coulter Counter inoperative as well as potentially causing a hazardous spill.

d. All counts are obtained using a 100 μ m aperture tube, and 0.5 ml sample volume. The sample is mixed by covering the top of a sample with parafilm, and inverting ~ three times.

e. To make a measurement, the aperture is inserted into the sample, the sample valve is opened and the Coulter Counter is allowed to reset. The valve is then closed to allow the sample to be drawn into the aperture and counted.

d. After field mussels have acclimated overnight, clearance rate measurements are taken. After one hour, water samples containing algal particles are taken from the outflow of each container, as well as from the overflow. A 20 ml vial is filled from each container. All samples are taken in rapid succession, including incoming samples from each delivery system. Algal counts are then measured on the Coulter Counter, as previously described. Two readings are taken per sample and the mean is recorded in a lab notebook (Table 2). This procedure is repeated three times at one hour intervals.

D. Respiration Rate Determinations

Respiration rate is defined as the amount of oxygen consumed by a mussel over a given period of time.

1. Radiometer Operation

PO₂ electrodes are used for measurement of oxygen within the respirometer (Fig. 6). Prior to each study, the PO₂ electrode undergoes a routine maintenance and calibration procedure:

- a. The electrode is disassembled and rinsed with DI. The tip of the electrode is gently cleaned with a bristled brush to remove accumulated debris and rinsed with DI.
- b. A new PO₂ membrane is then placed onto the outer casing, new electrolyte solution added, and the electrode reassembled back into the casing, taking care not to damage the membrane.
- c. The cleaned electrode is connected to a Radiometer oxygen meter and strip chart recorder are turned on. The electrode is then placed into zeroing solution and allowed to stabilize (a continuous straight line reading for at least 15 minutes, after which the zero setting is adjusted on both the Radiometer and the strip chart recorder.

2. Respirometer Vessels

Oxygen consumption is measured on individual mussels using 450 ml glass respirometer vessels (Fig. 4).

- a. Water flow through the respirometers is started and the chambers are allowed to flush with water. An air-driven magnetic stirrer located on the bottom of each chamber is turned on and the speed adjusted so that gentle mixing occurs.
- b. The respirometer lid is then clamped down, and the PO₂ electrode is gently inserted into the hole of the respirometer lid. After the electrode is secure, the inflow and outflow tubes are secured into the ground-glass surfaces. Sea water is flushed through the respirometer at a rate of 80 ml min⁻¹.
- c. The radiometer is adjusted to 100% saturation (160 mm Hg). To obtain a properly stabilized 100% saturation requires a continuous straight line reading for at least 20 minutes.

- d. Once the radiometer is calibrated and stable, a mussel is placed into the chamber.
- e. One of the glass tubes is removed to equalize the pressure as the lid is being secured. All air bubbles in the chamber are removed by tilting the vessel, allowing air bubbles and water to flow out of the hole.
- f. After at least 5 minutes under flow through conditions, the 3-way valves are closed to isolate the mussel. The mussel is allowed to respire for at least 20 minutes until a constant rate is observed (determined by a straight line on the strip chart recorder).
- g. The chamber is allowed to flush for at least 5 minutes before oxygen consumption is measured on the next mussel. This procedure is repeated until all mussels have been measured.

3. Respiration Rate Calculations

Respiration rates are calculated from the following formula:

$$R = (\text{MMHG} / 160 \text{ mmHg}) * (\text{SATO}_2) * (((\text{RESVOL}) - (\text{MUSVOL})) * 1 \text{ L} / 1000 \text{ ml}) * (60 \text{ min hr}^{-1} / \text{O}_2\text{TIME})$$

Where:

R	=	oxygen consumed by the mussel, (ml hr ⁻¹)
MMHG	=	change in partial pressure of O ₂ over time, (mm Hg min ⁻¹)
SATO ₂	=	oxygen saturation concentration of seawater at a given temperature, (ml l ⁻¹)
RESVOL	=	respiration vessel volume, (ml)
MUSVOL	=	volume of the mussel, (ml)
O ₂ TIME	=	time period of the measurement, (min)

- a. The variable MMHG is obtained by measuring the rate of decline (mm Hg min⁻¹) in O₂ concentration as recorded on the strip chart recorder through the following process:

1. Identify a portion of the decline with a straight edge and draw a line. This will serve as the hypotenuse of the triangle.
2. With a straight edge, mark another line where the hypotenuse and the chart paper grid intersect. This line should be 15 cm long from the point of intersection.
3. Complete the triangle by drawing a line to create a right angle. The chart paper units are counted and multiplied by 1.60 (converting the chart paper counts into mmHg). This value is recorded into the laboratory notebook.
4. Whole mussel volume is determined by measuring the amount of water that is displaced by the mussel when added to a container. The container fitted with a T-connector is filled to overflowing with sea water. The additional overflow resulting from the mussel addition is collected and measured with a 100 ml graduated cylinder to the nearest 0.5 ml and recorded into the laboratory

notebook (Table 3).

F Assimilation Efficiency Determinations

Assimilation efficiency is defined as the percentage of ingested food that is digested by the mussel. The prescribed algal concentration during feeding is selected to be below the level of pseudofeces production. This allows the assumption that all food that was consumed has passed through the mussel digestive system.

1. Mussels are returned to the clearance rate chamber after completion of the respiration rate measurements.
2. Residual fecal and algal material is removed from each feeding container by emptying and rinsing with sea water. Mussels are then allowed to feed overnight.
3. Fecal pellets are collected from each chamber with a Pasteur pipette, and filtered onto a 0.45 μm Nucleopore polycarbonate filter and rinsed with 1 N isotonic ammonium formate solution (48 g/2 L DI).
4. Slide the filter onto a watch glass and scrape the fecal material off with a plastic spatula onto pre-ashed, pre-weighed 1 cm^2 pans and dried at 60°C for 24 hr. Samples should be held in a desiccator prior to weighing.
5. Pellets and pans are weighed ($\pm 0.1 \mu\text{g}$) on a microbalance, ashed at 500°C for 4 hr, and re-weighed to determine the dry weight and ash weight for the feces (Table 4). A similar procedure is completed with the cultured algae to obtain the dry weight and ash weight of the food. A blank pan should be weighed after every nine sample pans to check the integrity of balance calibration. After all samples have been weighed, 10% are re-weighed to check the precision of the measuring technique.
6. The ash-free dry weight of food and feces is determined by the difference between the dry weight and the ash weight.
7. Assimilation efficiency (AE) is calculated according to Conover (1966) using the following formula:

$$\text{AE} = ((F-E)/(1-E)*F) * 100$$

Where: F = Ash-free dry weight:dry weight ratio of the food
E = Ash-free dry weight:dry weight ratio of the feces

F. Mussel Growth

1. The anterior-posterior length of each pre-measured (5-6 cm) mussel is determined with a vernier caliper ($\pm 0.1 \text{ mm}$). The pre-exposure length is subtracted from the post-exposure length to determine growth.

2. Mussel tissues are then excised, dried in pre-tared aluminum dishes at 100°C for 24 hr weighed to the nearest 0.01 g on a top loading balance. Ten percent are re-weighed to check the precision of the weighing technique.

IV. TROUBLE SHOOTING, QA/QC

1. Analytical balance calibration must be verified using standardized weights prior to use. If the calibration of an analytical balance is incorrect, a repair representative must be called in to recalibrate the balance.
2. The microbalance is calibrated prior to each use. The zero setting is first obtained, and the range expanded using a standardized weight. The sample weights are always less than the expanded range weight.
3. Other trouble shooting and QA/QC points are discussed in the Methods Section.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Scope for growth calculation.

- a. After all the physiological measurements have been made, raw data are entered into a computerized database from the laboratory notebook. A printout of the data is produced and checked for transcription errors.

- b. The SFG of each individual mussel is calculated through the following equation:

$$\text{SFG} = (C \times A) - R$$

where: SFG = scope for growth
C = energy consumed
A = assimilation efficiency
R = energy lost through respiration

- c. After all transcription errors have been corrected, the following energy conversions are incorporated to calculate SFG:

1 mg of Isochrysis = 4.5×10^7 cells
1 mg of Isochrysis = 19.24 J (Nelson, 1990)
1 ml O₂ respired = 20.08 J (Crisp, 1971).

These conversions are achieved on the computer through an automated series of command files (Table 5), where the SFG values are calculated for each mussel.

- d. Statistical analyses for an experiment are conducted through SAS using a one-way analysis of variance (ANOVA) to test for statistical differences among stations (Snedecor

and Cochran, 1980). If the ANOVA test is significant ($P=0.05$), Tukey's Studentized Range Test is applied to determine where the differences lie. The SAS commands are listed in Table 6.

VI. REFERENCES

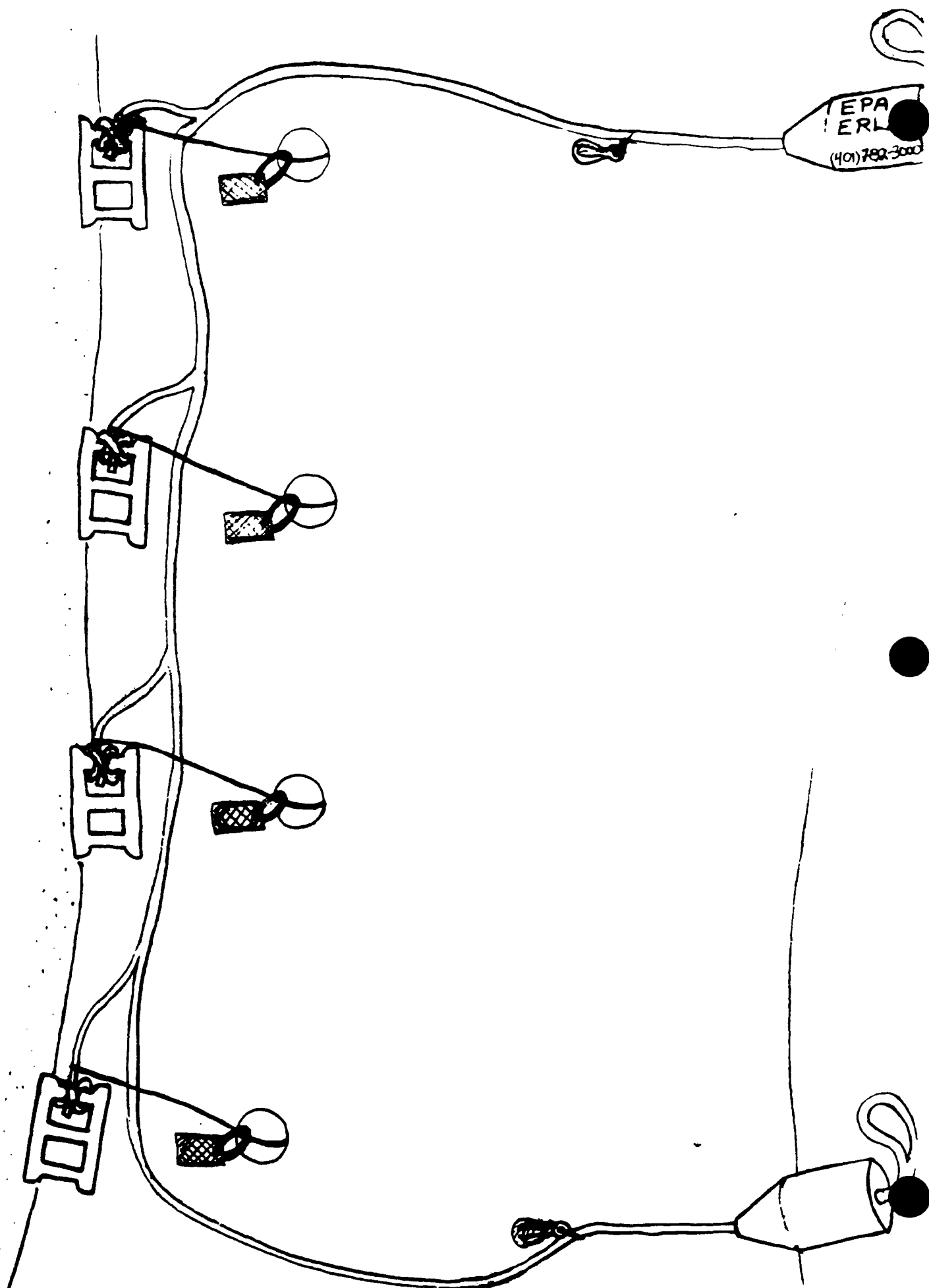
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**ERL-N STANDARD OPERATING PROCEDURE
GROWTH AND SCOPE FOR GROWTH MEASUREMENTS
WITH *Mytilus edulis***

ERL-N SOP 1.03.013
REVISION 0
FEBRUARY 1991
PAGE 17 OF 29

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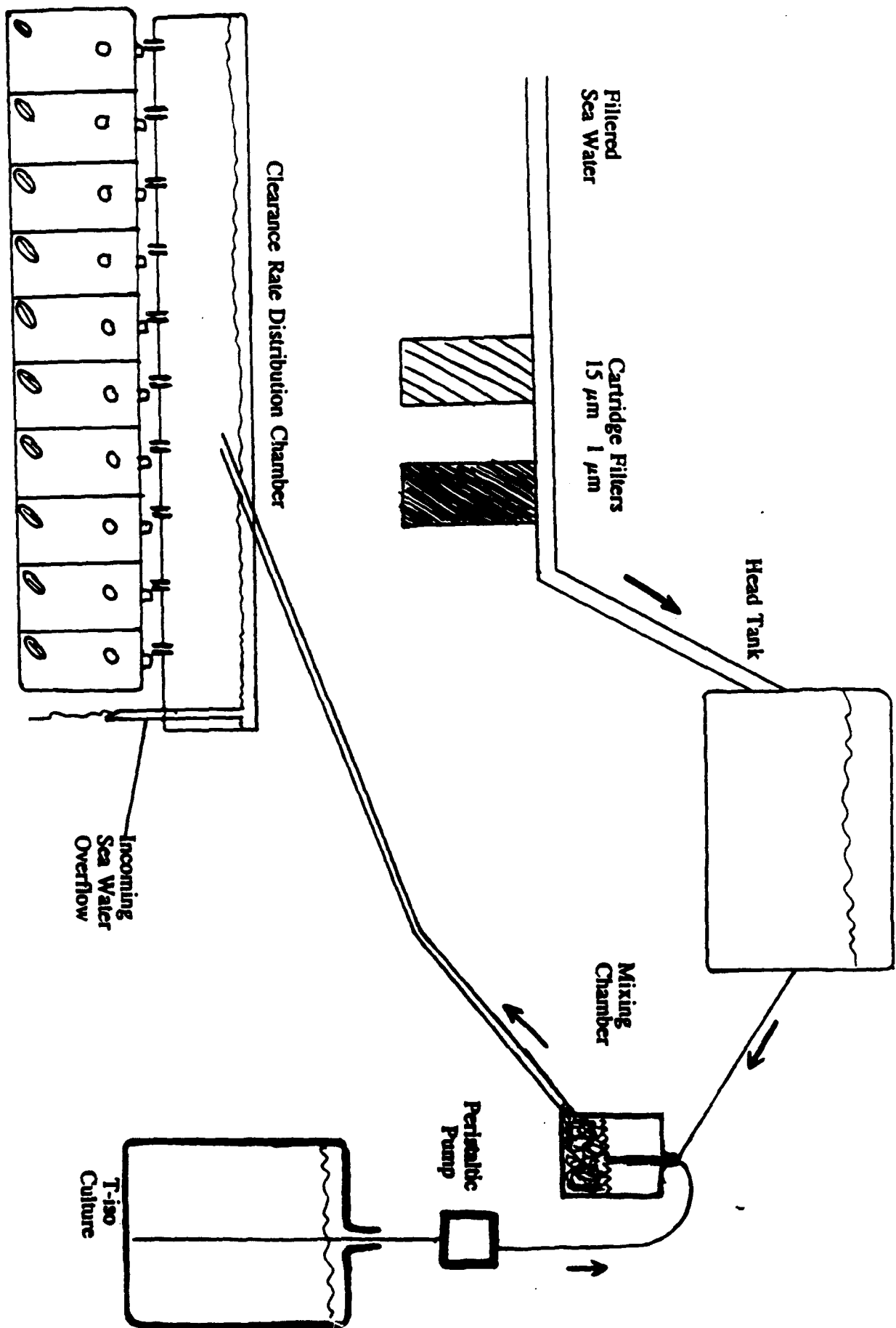


Figure 2. Flow diagram of the clearance rate distribution system.

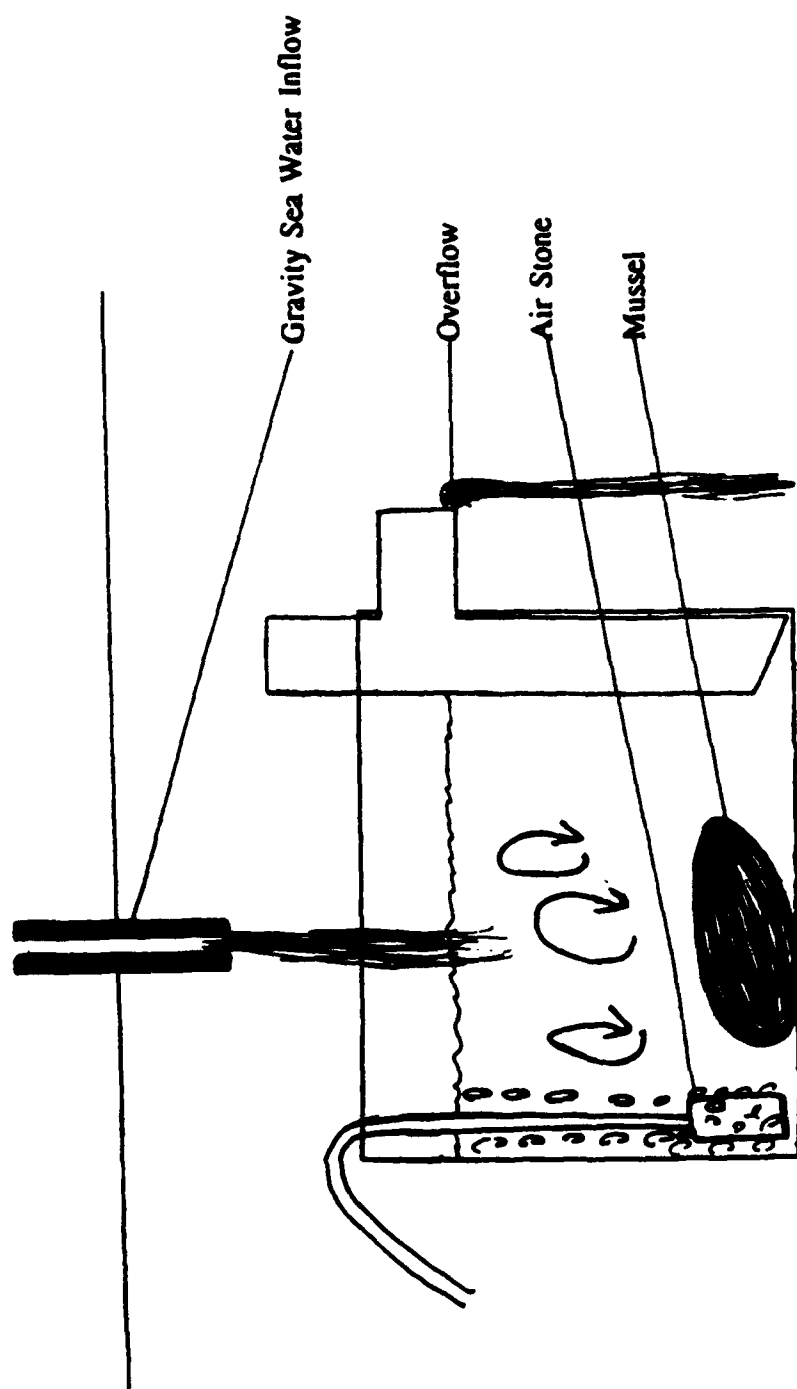


Figure 3. The clearance rate chamber.

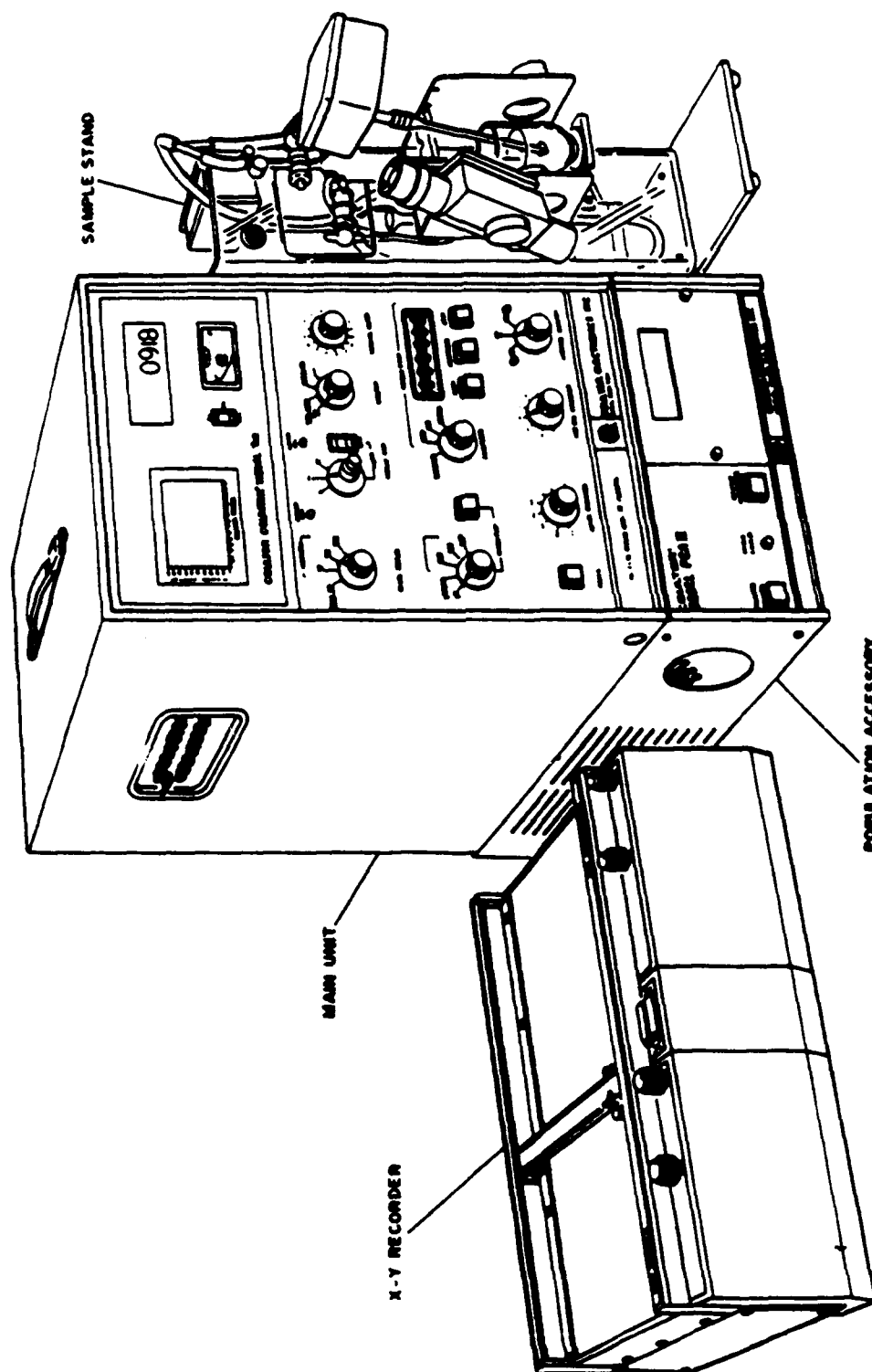


Figure 4. The Coulter Counter Particle Counter (Model TAIL).

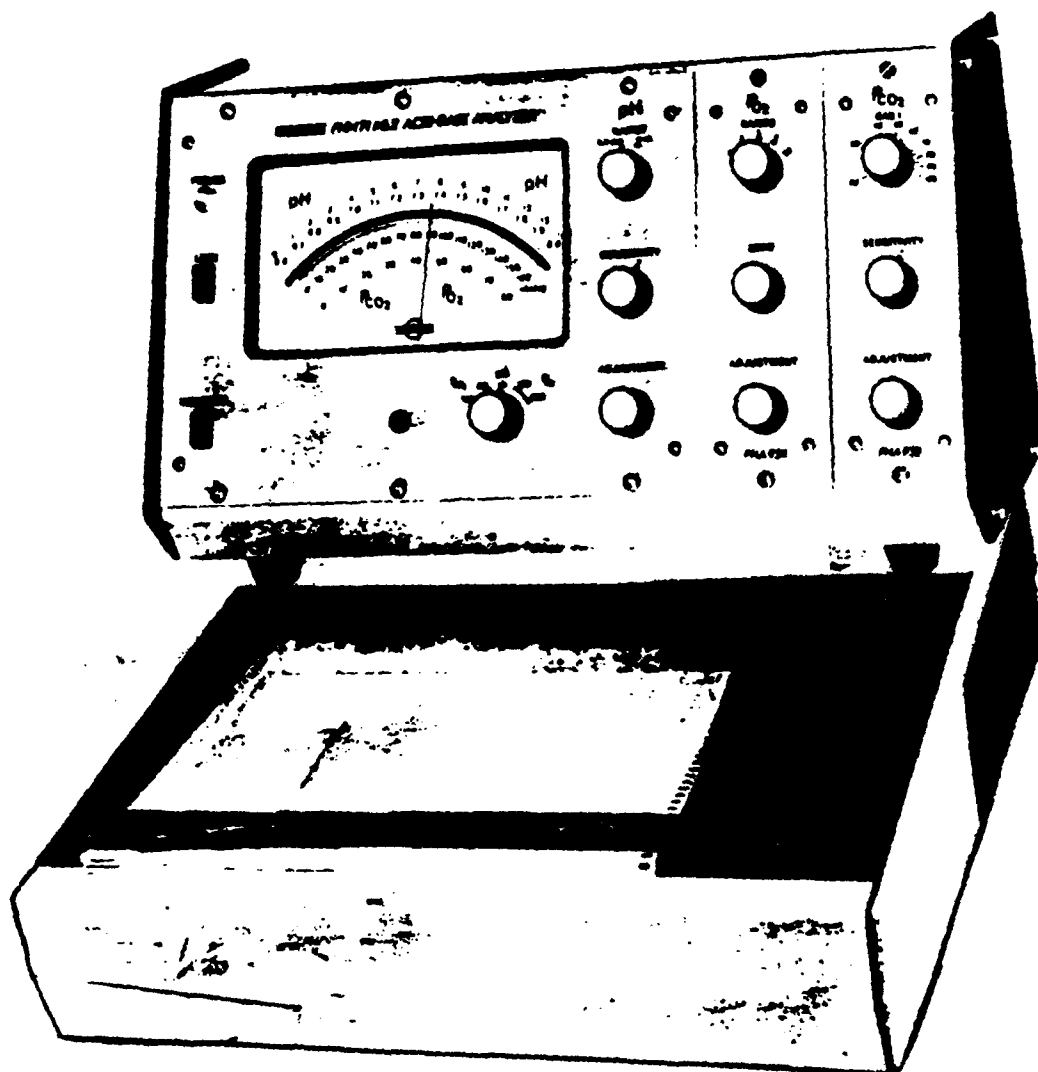


Figure 6. The radiometer.

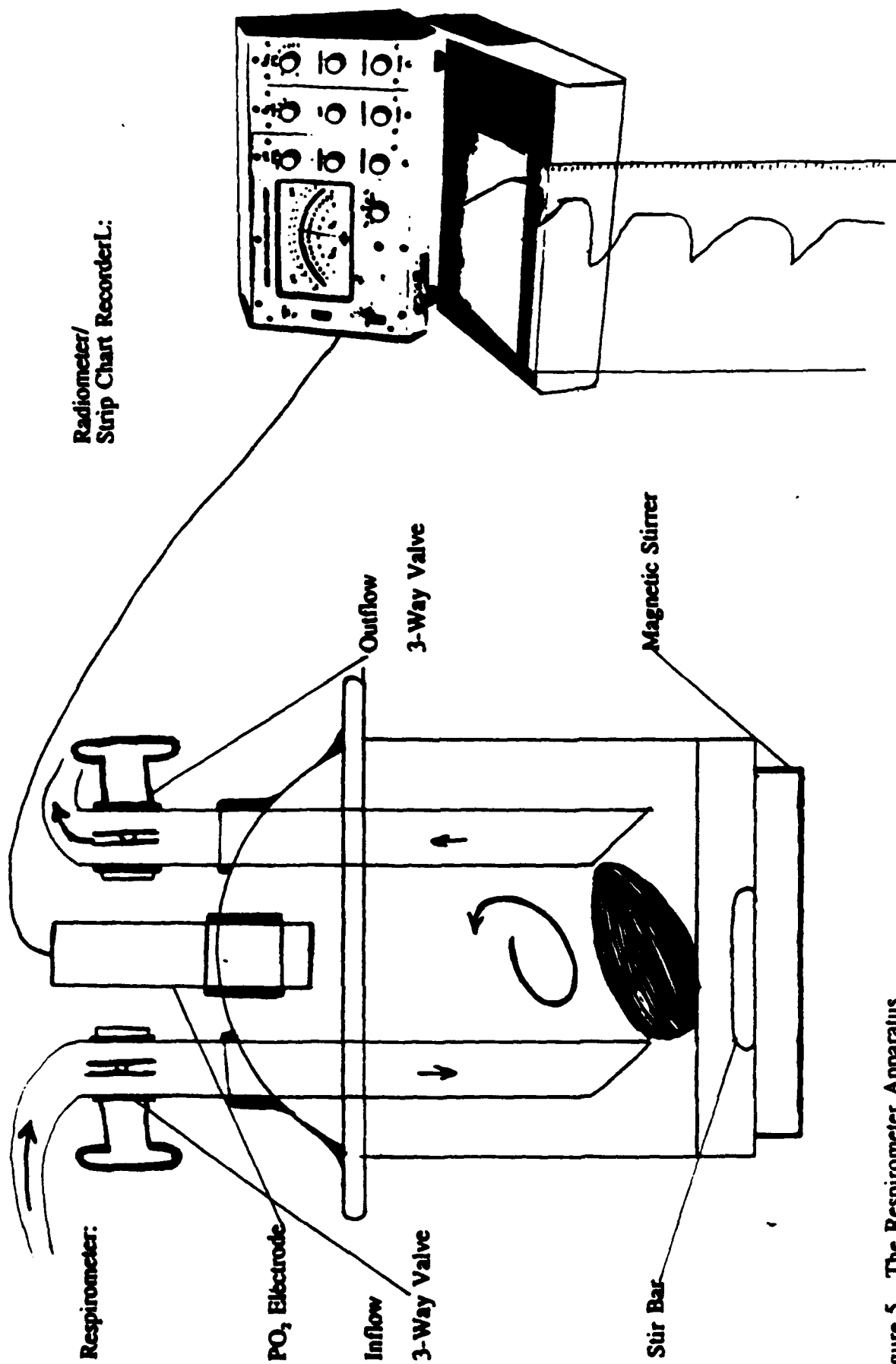
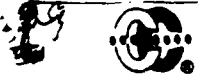


Figure 5. The Respirometer Apparatus.

Table 1. Coulter Counter Model TA Series Worksheet.



COULTER COUNTER® MODEL TA SERIES Worksheet

SAMPLE									
ELECTROLYTE					DISPERSANT				
EQUIPMENT	SERIAL	Aper. Dia.	Ser. No.	CALIBRATION MATERIAL	Part Dia.	W	mA	A.	
ORGANIZATION									
OPERATOR	DATE								

APER. DIAM.	260			
PART. DIAM.	0.21	0.21		
W	7	6		
mA				
A ₂		12.2		

$$\frac{A_2}{A_1} = \left(\frac{d_1}{d_2}\right)^2 (W_1 \cdot W_2)$$

$$\frac{A_2}{A_1} = \left(\frac{d_1}{d_2}\right)^2 \text{ WHEN } W_1 = W_2$$

SAMPLE DATA

Geometric Mean μ^3	Volume μ^3	Diameter μ	CHANNEL NO. (W)	
00575	004001	100		
.0115	.008161	.250		
.0231	.01636	.318		
.0462	.03272	.397		
.0925	.06545	.500		
.1851	.1309	.630		
.3702	.2618	.794		
.7405	.5236	1.00		
1.481	1.047	1.26		
2.962	2.094	1.59		
5.924	4.188	2.00		
11.85	8.378	2.82	1	
23.70	16.76	3.17	2	
47.39	33.51	4.00	3	2
94.78	67.02	5.04	4	3
189.6	134.0	6.36	5	4
379.1	268.1	8.00	6	5
758.3	536.2	10.00	7	6
1516	1072	12.7	8	7
3033	2145	16.0	9	8
6066	4290	20.3	10	9
12.13 x 10 ³	6570	25.4	11	10
24.27 x 10 ³	17.16 x 10 ³	32.0	12	11
48.54 x 10 ³	34.31 x 10 ³	40.3	13	12
97.18 x 10 ³	68.63 x 10 ³	50.6	14	13
194.4 x 10 ³	137.3 x 10 ³	64.0	15	14
388.7 x 10 ³	274.6 x 10 ³	80.0	16	15
777.4 x 10 ³	549.0 x 10 ³	101.6	17	16
1.555 x 10 ⁴	1.098 x 10 ⁴	128		
3.109 x 10 ⁴	2.196 x 10 ⁴	161		
6.218 x 10 ⁴	4.392 x 10 ⁴	203		
12.44 x 10 ⁴	8.784 x 10 ⁴	256		
24.88 x 10 ⁴	17.57 x 10 ⁴	322		
49.76 x 10 ⁴	35.14 x 10 ⁴	400		
99.50 x 10 ⁴	70.27 x 10 ⁴	512		
199.0 x 10 ⁴	140.5 x 10 ⁴	640		
398.0 x 10 ⁴	281.1 x 10 ⁴	812		
796.0 x 10 ⁴	562.2 x 10 ⁴	1024		
1592 x 10 ⁴	1124 x 10 ⁴	1280		
3184 x 10 ⁴	2248 x 10 ⁴	1624		
6368 x 10 ⁴	4496 x 10 ⁴	2048		
1274 x 10 ⁴	8992 x 10 ⁴	2592		

Table 2. Data notebook - Clearance Rates.

Allen Harbor Phase II; Deploy. #1 / ⁴² ~~calat~~ = 900430
 Temp = 12 / ~~calat~~ = 900501
 start 2/10/8

SRG	SRGNUM	SFGNUM	STAND	Rep	1130	1140	1200	Rinc
115199	300840	3701	AM	1	9245	9055	8680	
"	"	02		1	8831	7973	7335	Rinc1 =
300	841	03		2	8209	6642	6163	9013
"	"	04		2	8144	7839	6513	
301	842	05		3	6560	5310	5572	Rinc2 =
"	"	06		3	6576	5756	5028	9096
302	843	07		4	8819	8995	8606	
"	"	08		4	7974	6864	6439	Rinc3 =
								8769
198311	300849	3711	TTM	1	8127	7528	7297	
"	"	12		1	6930	6500	5806	
312	899	13		2	8355	7513	7194	
"	"	14		2	7380	6704	6157	
313	850	15		3	7096	5888	5333	
"	"	16		3	7387	6544	6157	
314	851	17		4	6221	5542	5767	
"	"	18		4	9165	6649	6879	
19305	300849	3721	MV	1	4753	5368	4951	
"	"	22		1	6446	5877	7131	
306	885	23		2	9278	5406	5055	Rinc1 =
"	"	24		2	4233	3749	4509	9167
307	846	25		3	7465	5887	5826	
"	"	26		3	7349	7305	6987	Rinc2 =
308	847	27		4	5662	5723	5626	9246
"	"	28		4	4715	4564	4312	
								Rinc3 =
19317	300852	3731	LMB	1	7119	6965	6379	9287
"	"	32		1	4933	4999	4587	
318	853	33		2	3738	2572	2394	
"	"	34		2	4813	4319	5824	
319	854	35		3	5011	5257	4780	
"	"	36		3	6314	6265	3966	
320	855	37		4	5443	5610	4765	
"	"	38		4	5553	5586	5552	

Table 3. Data notebook - Respiration rates and growth.

43

Allen Harbor Respiration: 900500 - 100503

Temp = 12, ReVol = 430, SATD = 6.24

Massum	SEGum	mmHg	musical	Length	wt
1	3701	8.0	15	5.23	2.04
2	02	8.3	16	5.87	2.28
3	03	8.3	15	5.83	1.83
4	04	9.0	17	5.86	1.86
5	05	8.0	15	5.65	1.83
6	06	8.3	15	5.61	2.03
7	07	10.7	16	5.88	2.25
8	08	11.2	17	5.77	2.04
11	3711	7.5	16	5.82	2.10
12	12	6.7	16	5.86	2.16
13	13	10.1	15	5.61	2.24
14	14	8.8	17	5.63	2.58
15	15	10.4	16	5.89	2.37
16	16	7.2	13	5.69	2.16
17	17	7.5	15	5.69	2.00
18	18	7.5	17	5.83	2.16
21	3721	9.1	15	5.46	1.67
22	22	9.1	13	5.58	1.67
23	23	7.5	13	5.62	1.87
24	24	7.5	13	5.55	1.61
25	25	8.5	13	5.65	1.52
26	26	8.0	14	5.68	1.57
27	27	7.5	13	5.60	1.56
28	28	4.7	13	5.47	1.25
31	3731	8.0	14	5.92	1.67
32	32	7.5	15	5.66	1.54
33	33	8.5	15	5.98	1.68
34	34	8.7	14	5.77	1.73
35	35	7.2	14	5.82	1.10
36	36	8.0	13	5.48	1.39
37	37	7.5	14	5.66	1.32
38	38	8.0	15	5.52	1.54

Table 4. Data notebook - Assimilation Efficiency.

Assimilation Efficiency: New Harbor, Phase II							42
900503 - 900504							
<u>musnum</u>	<u>SFGNUM</u>	<u>ANUM</u>	<u>parent</u>	<u>Dayton</u>	<u>Ashpan</u>	<u>mortality</u>	
21-22	3721-22	17	15.38	16.49	15.72	0	
23-24	23-24	29	15.03	16.49	15.53	0	
25-26	25-26	30	15.36	16.53	15.74	73	
27-28	27-28	31	15.62	18.01	16.54	0	
31-32	3731-32	32	13.37	15.68	14.14	0	sanctuary
33-34	33-34	33	16.11	17.53	16.57	0	all 78
35-36	35-36	34	15.69	16.69	16.01	0	
37-38	37-38	35	14.52	16.56	15.05	0	
100503-1-2	3701-02	20	16.06	19.35	17.06	0	
3-4	03-4	21	16.85	18.92	17.36	0	
5-6	5-6	22	16.67	19.18	17.33	0	
7-8	7-8	23	14.10	16.36	14.77	0	
11-12	3711-12	24	15.58	17.61	16.17	0	
13-14	13-14	25	13.41	16.57	14.82	1	
15-16	15-16	26	16.40	18.02	16.92	0	
17-18	17-18	27	15.96	18.33	16.58	0	

Table 5. Command files of data transformations.

```

.....
THIS COMMAND FILE WILL BEGIN THE CALCULATION OF THE SCOPE FOR
GROWTH INDEX FOR MYTILUS EDULIS. IT WILL FIRST DELETE THE
TEMPORARY SAVE FILES THEN PROCEED TO THE DATMAN FILE PROMPT.DTM.
THIS FILE WILL COMPLETE THE TRANSFORMATION OF ALL THE RAW DATA.
INSERT THEM INTO THE DATA BASE SFG. ALL OF THE DATA INSERTED
INTO SFG WILL BE PRINTED OUT SO THAT A HARD COPY IS AVAILABLE.
.....
DELETE CLEAR.DTM;
DELETE ABSORP.DTM;
DELETE OXYGEN.DTM;
DELETE ENERGY.DTM;
DATMAN PROMPT.DTM;
DELETE CLEAR.DTM;
DELETE ABSORP.DTM;
DELETE OXYGEN.DTM;
DELETE ENERGY.DTM;

```

```

.....
THE RAW CLEARANCE RATE, ABSORPTION EFFICIENCY, RESPIRATION RATE,
AND AMMONIA EXCRETION RATE DATA WILL NOW BE TRANSFORMED TO ALLOW
THE CALCULATION OF THE SCOPE FOR GROWTH INDEX
.....
SEARCH SFG FROM CLRCAL.INC

```

```

CINC1=((RINC1-1000)*.2*.2.5)+RINC1)*.2
CINC2=((RINC2-1000)*.2*.2.5)+RINC2)*.2
CINC3=((RINC3-1000)*.2*.2.5)+RINC3)*.2
COUT1=((ROUT1-1000)*.2*.2.5)+ROUT1)*.2
COUT2=((ROUT2-1000)*.2*.2.5)+ROUT2)*.2
COUT3=((ROUT3-1000)*.2*.2.5)+ROUT3)*.2
CLR1=((CINC1-COUT1)/COUT1)*FLOWRT
CLR2=((CINC2-COUT2)/COUT2)*FLOWRT
CLR3=((CINC3-COUT3)/COUT3)*FLOWRT
IF (CLR1<0) CLR1=0
IF (CLR2<0) CLR2=0
IF (CLR3<0) CLR3=0
AVGCLR=((CLR1+CLR2+CLR3)/3)
PRCLN1=((CINC1-COUT1)/CINC1)*FLOWRT
PRCLN2=((CINC2-COUT2)/CINC2)*FLOWRT
PRCLN3=((CINC3-COUT3)/CINC3)*FLOWRT
IF (PRCLN1<0) PRCLN1=0
IF (PRCLN2<0) PRCLN2=0
IF (PRCLN3<0) PRCLN3=0
AVGPCLR=((PRCLN1+PRCLN2+PRCLN3)/3)
COMMAND CLRCAL2.INC
INPUT INCLUDE.DTM

```

```

.....
THE TRANSFORMED CLEARANCE RATE DATA WILL NOW BE SAVED ON CLEAR
.....
SAVE ON CLEAR SFGNUM,CLR1,CLR2,CLR3,AVGCLR,AVGPCLR
.....
SEARCH SFG FROM ABCAL.INC

```

```

FECDT=FECDT.FILTIMT
ASHWT=ASHWT.FILTIMT
AFDWS=((FECDT.ASHWT)/FECDT
ABSEFF=((AFDWT-AFDWS)/AFDWS)*AFDWS
IF (ABSEFF<0) ABSEFF=0
IF (ABSEFF<0) ABSEFF=0
COMMAND ABCAL2.INC

```

```

.....
THE TRANSFORMED ASSIMILATION EFFICIENCY DATA WILL NOW BE SAVED ON ABSORP
.....
SAVE ON ABSORP SFGNUM,FECDT,ASHWT,ABSEFF,AFDWS
.....
SEARCH SFG FROM OZCAL.INC
.....
VAR1=((RMSG/160)*SATO2)
VAR2=((RESVOL-RUSVOL)/1000)
VAR3=((60/02TIME)
RLOZNR=((VAR1*VAR2)*VAR3)
COMMAND OZCAL2.INC
INPUT INCLUDE.DTM

```

```

.....
THE TRANSFORMED CLEARANCE RATE DATA IS NOW BEING
ENTERED INTO DATA BASE SFG
.....
INSERT SFG
INPUT CLEAR.DA
FORMAT CLEAR.NA
OUTPUT
END
.....
THE TRANSFORMED ASSORPTION EFFICIENCIES ARE NOW
BEING ENTERED INTO DATA BASE SFG
.....

```

```

INSERT SFG
INPUT ABSORP.DA
FORMAT ABSORP.NA
OUTPUT
END
.....
THE TRANSFORMED RESPIRATION RATE DATA IS NOW BEING
ENTERED INTO DATA BASE SFG
.....
INSERT SFG
INPUT OXYGEN.DA
FORMAT OXYGEN.NA
OUTPUT
END
.....
SEARCH SFG FROM SFGCAL.INC

```

```

.....
THE FINAL SCOPE FOR GROWTH VALUES ARE NOW BEING
INSERTED INTO DATA BASE SFG
.....
INSERT SFG
INPUT ENERGY.DA
FORMAT ENERGY.NA
OUTPUT
END

```

```

.....
THE TRANSFORMED SFG DATA WILL NOW BE SAVED ON ENERGY
.....
SAVE ON ENERGY SFGNUM,JSFG
.....
DATMAN CONENG.DTM

```

```

JCLR=AVGPCLR*LABENG
JABS=JCLR*ABSEFF
JRESR=JABS*20.06
JSFG=JABS*(JRESR)
COMMAND SFGCAL2.INC
INPUT INCLUDE.DTM

```

Table 6. SAS commands used in SFG Statistical Analyses.

```
PROC SORT; BY STATID;
PROC PRINT;
PROC MEANS; BY STATID;
PROC PLOT;
PLOT AVGCLR*DRYWT=STATID;
PLOT ABSEFF*DRYWT=STATID;
PLOT MLO2HR*DRYWT=STATID;
PLOT JSFG*DRYWT=STATID;
PROC REG;
MODEL AVGCLR=DRYWT;
MODEL ABSEFF=DRYWT;
MODEL MLO2HR=DRYWT;
PROC REG; BY STATID;
MODEL JSFG=DRYWT;
PROC GLM;
CLASSES STATID;
MODEL JSFG = STATID DRYWT DRYWT*STATID;
LSMEANS STATID/STDERR PDIFF;
PROC SORT; BY STATID REP;
PROC MEANS; BY STATID REP;
VAR AVGCLR ABSEFF MLO2HR JSFG DRYWT LENGTH;
OUTPUT OUT=REPL MEAN=MCLR MABS MMLO2 MJSFG MDRYWT MLEN;
PROC PRINT;
PROC GLM;
CLASSES STATID;
MODEL MCLR MABS MMLO2 MJSFG MLEN = STATID MDRYWT MDRYWT*STATID;
LSMEANS STATID/STDERR PDIFF;
PROC GLM;
CLASSES STATID;
MODEL MCLR MABS MMLO2 MJSFG MDRYWT MLEN = STATID;
MEANS STATID/DUNCAN TUKEY;
```

ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE
ENUMERATION OF *Clostridium perfringens*
IN MARINE WATERS

ERL-N SOP 1.03.014
REVISION 0
MARCH 1991
PAGE 1 OF 5

POINT OF CONTACT:

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I. OBJECTIVE

Clostridium perfringens is often found in the intestines of humans and animals as part of the normal microbiota and is therefore widely distributed in soil and water. This spore-forming microorganism is capable of surviving for long periods of time in the environment, and thus is often used in the detection of fecal contamination that is several years old. Clostridium perfringens is most often encountered in gangrene, but is also known to produce a common type of food poisoning as well as a more serious necrotizing infection of the small bowel. Considering this pathogenicity for man, enumeration of Clostridium perfringens spores in marine waters using the membrane filtration method is often performed as a means of determining the sanitary quality of marine environments and the waters which may impact marine environments.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware

- 2 liter Erlenmeyer flask
- 9 x 50 mm petri dishes (Falcon)
- 16 x 150 mm test tubes
- 1 liter polypropylene bottles
- 10 ml sterile pipettes
- Cornwall syringe

*Sterilized at 121°C for 15 minute

B. Media and Diluent

- Media Ingredients:

- Tryptose (Difco)
- Yeast Extract (Difco)
- Sucrose (Difco)
- L-cysteine Hydrochloride (Baker Analyzed)
- Bromocresol Purple (Difco)
- 10N Sodium Hydroxide (Baker Analyzed)

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE
ENUMERATION OF *Clostridium perfringens*
IN MARINE WATERS**

ERL-N SOP 1.03.014
REVISION 0
MARCH 1991
PAGE 2 OF 5

- D-cycloserine (Sigma)
 - Polymyxin B Sulfate (Sigma)
 - Ferric Chloride 6 Hydrate (Baker Analyzed)
 - Phenolphthalein Diphosphate (Sigma)
 - Ammonium Hydroxide (Sigma)
 - Deionized Water
- Diluent Ingredients:
- Sodium chloride (Baker Analyzed)
 - Phosphate buffer (Baker Analyzed)
 - Deionized water

C. Equipment

- Heating mantle with magnetic stirrer
- pH meter
- Analytical balance
- Alcohol burner
- Filter holders (Gelman Science #4468321)
- Ultraviolet light sterilizer unit (Millipore #XX6370000)
- Stainless steel filter holder manifold (Whatman MS-6)
- Membranes 0.7 micron (Millipore HCW6047S3)
- Membrane forceps
- Anaerobic incubator 44.5-45.0°C
- Vacuum unit

III. METHODS

A. Membrane Filtration Media: mCP Agar

1. Weigh 30.0 g of tryptose, 20.0 g of yeast extract, 5.0 g of sucrose, 1.0 g of l-cysteine hydrochloride and 0.04 g of bromocresol purple into a 2 liter Erlenmeyer flask, add 980.0 ml of deionized water and mix well.
2. Adjust the pH to 7.6 using 10N sodium hydroxide.
3. Add 15.0 g of agar and boil briefly to dissolve.
4. Autoclave at 121°C for 15 minutes.
5. Temper the media to 45°C for approximately 30 minutes.
6. Aseptically add:
 - 0.04 g of D-cycloserine
 - 0.025 g of polymyxin B sulfate

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE
ENUMERATION OF *Clostridium perfringens*
IN MARINE WATERS**

**ERL-N SOP 1.03.014
REVISION 0
MARCH 1991
PAGE 3 OF 5**

- 2.0 ml of a 4.5% ferric chloride 6 hydrate solution
- 20.0 ml of a 0.5% phenolphthalein solution

7. Dispense the medium in 4.5 ml aliquots into 9 x 50 mm petri dishes.
8. Store mCP agar plates inverted at 4°C in the dark for up to one month.

B. Diluent Preparation: Phosphate Buffered Saline (PBS)

1. Weigh 8.5 g of sodium chloride into a polypropylene bottle.
2. Add 1.0 liter of deionized water and stir to dissolve.
3. Add 1.25 ml of phosphate buffer and stir well.
4. Autoclave at 121°C for 15 minutes.
5. Store at room temperature for up to one month.

C. Sample Preparation

1. All water samples were shaken vigorously (25 complete up and down movements of 1ft/7sec) prior to transfer of sample to PBS dilution or enrichment tube.

D. Sample Analyses

1. Filter holders are sterilized using the ultraviolet light sterilization unit and inserted into the filter manifold.
2. Filter holders are loaded with membrane filters.
3. Approximately 50.0 ml of sterile PBS is added to the filter holder prior to adding samples of less than or equal to 20.0 ml volumes. Addition of phosphate buffered saline is not required for sample volumes greater than 20.0 ml.
4. An aliquot of sample, or sample dilution, is added to the filter holder.
5. Vacuum is applied to the receiver flask.
6. The walls of the funnel are rinsed with sterile PBS.
7. Vacuum is applied to draw the rinse buffer through.
8. The filter is removed with sterile forceps and then placed on a mCP agar plate.
9. Plates are incubated anaerobically at 44.5-45°C for 18 hours.

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE
ENUMERATION OF *Clostridium perfringens*
IN MARINE WATERS**

ERL-N SOP 1.03.014
REVISION 0
MARCH 1991
PAGE 4 OF 5

10. Typical large, flat, yellow colonies are scored.
11. Plates are held over fresh ammonium hydroxide for approximately 20-30 seconds.
12. *Clostridium perfringens*, which turn magenta, are scored.

IV. TROUBLE SHOOTING

1. Thoroughly rinse the walls of the filter holder prior to removing membrane filters.
2. Sterilize the membrane forceps between samples, and dilutions of samples, to avoid contamination.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data.
2. Data are used in conjunction with other indicator tests to determine sanitary quality.

VI. REFERENCES

- American Public Health Association, Inc. 1970. Recommended Procedures for the Examination of Sea Water and Shellfish. Washington, DC. p. 1-105.
- Bisson, J.W., V.J. Cabelli. 1978. Membrane filter enumeration method for *Clostridium perfringens*. J. Appl. Environ. Microbiol. 37:55-66.

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE ENUMERATION
OF ENTEROCOCCI IN MARINE WATERS**

ERL-N SOP 1.03.015
REVISION 0
MARCH 1991
PAGE 1 OF 5

POINT OF CONTACT:

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I. OBJECTIVE

Enterococci, including Streptococcus faecalis and Streptococcus faecium, are found in the intestines of humans and other animals, where they behave as commensals of limited pathogenic potential. Enterococci may cause mild gastrointestinal disease or less commonly, infections of the tissues of the intestines. Because these organisms are associated with fecal wastes of man and animals, they can be isolated from contaminated waters containing such waste. The use of a selective and differential medium allows for the enumeration of this fecal pollution indicator in marine waters using the membrane filtration method.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware

- 2 liter Erlenmeyer flask
- 9 x 50 mm petri dishes (Falcon)
- 16 x 150 mm test tubes
- 1 liter polypropylene bottles
- 10 ml sterile pipettes
- Cornwall syringe

*Sterilized at 121°C for 15 minutes

B. Media and Diluent

- Media Ingredients:

- mE Agar (Difco)
- Nalidixic Acid (Sigma)
- 10N Sodium Hydroxide (Sigma)
- 2,3,5 Triphenyltetrazolium Chloride (Sigma)
- Indoxyl-B-D-Glucoside (Sigma)
- 95 % Ethanol (Sofecia, SA)
- Sterile deionized water

- Diluent Ingredients:

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE ENUMERATION
OF ENTEROCOCCI IN MARINE WATERS**

ERL-N SOP 1.03.015
REVISION 0
MARCH 1991
PAGE 2 OF 5

- Sodium chloride (Baker Analyzed)
- Phosphate buffer (Baker Analyzed)
- Deionized water

C. Equipment

- Heating mantle with magnetic stirrer
- pH meter
- Analytical balance
- Alcohol burner
- Filter holders (Gelman Science #4468321)
- Ultraviolet light sterilizer unit (Millipore #XX6370000)
- Stainless steel filter holder manifold (Whatman MS-6)
- Membranes 0.7 micron (Millipore HCW6047S3)
- Membrane forceps
- Air incubator 41°C
- Vacuum unit

III. METHODS

A. Membrane Filtration Media: mE Agar

1. Weigh 71.2 g of mE agar into a 2 liter Erlenmeyer flask.
2. Add 1.0 liter of deionized water and boil with continuous stirring.
3. Autoclave at 121°C for 15 minutes.
4. Temper the media to 45°C for approximately 30 minutes.
5. Aseptically add:
 - 0.24 g of nalidixic acid dissolved in 0.3 ml of sterile deionized water and 0.2 ml of 10N sodium hydroxide
 - 0.75 g of indoxyl-B-D-glucoside dissolved in 5.0 ml of 95% ethanol and 5.0 ml of sterile deionized water
 - 0.2 g of 2,3,5 triphenyltetrazolium chloride
6. Adjust the pH to 7.1 ± 0.01 .
7. Dispense the medium into 9 x 50 mm petri dishes in 4.5 ml aliquots.
8. Store mE agar plates inverted at 4°C in the dark for up to one month.

B. Diluent Preparation: Phosphate Buffered Saline (PBS)

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE ENUMERATION
OF ENTEROCOCCI IN MARINE WATERS**

ERL-N SOP 1.03.015
REVISION 0
MARCH 1991
PAGE 3 OF 5

1. Weigh 8.5 g of sodium chloride into a polypropylene bottle.
2. Add 1.0 liter of deionized water and stir to dissolve.
3. Add 1.25 ml of phosphate buffer and stir well.
4. Autoclave at 121°C for 15 minutes.
5. Store at room temperature for up to one month.

C. Sample Preparation

All water samples were shaken vigorously (25 complete up and down movements of 1ft/7sec) prior to transfer of sample to PBS dilution or enrichment tube.

D. Sample Analyses

1. Filter holders are sterilized using the ultraviolet light sterilization unit and inserted into the filter manifold.
2. Filter holders are loaded with membrane filters.
3. Approximately 50.0 ml of sterile PBS is added to the filter holder prior to adding samples of less than or equal to 20.0 ml volumes. Addition of phosphate buffered saline is not required for sample volumes greater than 20.0 ml.
4. An aliquot of sample, or sample dilution, is added to the filter holder.
5. Vacuum is applied to the receiver flask.
6. The walls of the funnel are rinsed with sterile PBS.
7. Vacuum is applied to draw the rinse buffer through.
8. The filter is removed with sterile forceps and then placed on a mE plate.
9. Plates are incubated inverted at 41°C for 48 hours.
10. Typical colonies, those with pink centers, gray fringes and blue halos are scored enterococci.

IV. TROUBLE SHOOTING

1. Thoroughly rinse the walls of the filter holder prior to removing membrane filters.
2. Sterilize the membrane forceps between samples and dilutions of samples to avoid

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE ENUMERATION
OF ENTEROCOCCI IN MARINE WATERS**

**ERL-N SOP 1.03.015
REVISION 0
MARCH 1991
PAGE 4 OF 5**

contamination.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data.
2. Data are used in conjunction with other indicator tests to determine sanitary quality.

VI. REFERENCES

- American Public Health Association, Inc. 1970. Recommended Procedures for the Examination of Sea Water and Shellfish. Washington, DC. p. 1-105.
- Levin, M.A., S.R. Fischer, V.J. Cabelli. 1975. Membrane Filter Technique for Enumeration of Enterococci in Marine Waters. Appl. Microbiol. 30:66-71.

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE
ENUMERATION OF FECAL COLIFORMS AND
Escherichia coli IN MARINE WATERS**

ERL-N SOP 1.03.016
REVISION 0
MARCH 1991
PAGE 1 OF 5

POINT OF CONTACT:

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I. OBJECTIVE

Fecal coliforms, including *Escherichia coli*, are found in the intestinal tract of man and both warm- and cold-blooded animals. For this reason, they are often employed as indicators of fecal contamination. The National Shellfish Sanitation Program (NSSP) requires that a sanitary survey be performed of every potential shellfish growing area. The sanitary survey is a microbiological evaluation of all actual and potential pollution sources and environmental factors which may influence the water quality in shellfish growing areas. Although the membrane filtration method is not approved for shellfish water classification, it is often used in pollution load assessment. Enumeration of fecal coliforms and *Escherichia coli* using the membrane filtration method is a means of determining the sanitary quality of marine environments and the potential pollution sources which may impact marine environments.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware

- 2 liter Erlenmeyer flask
- 9 x 50 mm petri dishes (Falcon)
- 16 x 150 mm test tubes
- 1 liter polypropylene bottles
- 10 ml sterile pipettes
- Cornwall syringe*

*Sterilized at 121°C for 15 minute

B. Media and Diluent

- Media Ingredients:

- mTEC Agar (Difco)
- Deionized water

- Reagent Ingredients:

- Urea (Baker Analyzed)
- Phenol Red (Difco)

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE
ENUMERATION OF FECAL COLIFORMS AND
Escherichia coli IN MARINE WATERS**

ERL-N SOP 1.03.016
REVISION 0
MARCH 1991
PAGE 2 OF 5

- Hydrochloric Acid (Mallincrodt)
- Sterile deionized water
- Diluent Ingredients:
 - Sodium chloride (Baker Analyzed)
 - Phosphate buffer (Baker Analyzed)
 - Deionized water

C. Equipment

- Heating mantle with magnetic stirrer
- pH meter
- Analytical balance
- Alcohol burner
- Filter holders (Gelman Science #4468321)
- Ultraviolet light sterilizer unit (Millipore #XX6370000)
- Stainless steel filter holder manifold (Whatman MS-6)
- Filter pads (Millipore AP100U7S1)
- Membranes 0.7 micron (Millipore HCW6047S3)
- Membrane forceps
- Air incubator 44°C
- Styrofoam blocks
- Vacuum unit

III. METHODS

A. Membrane Filtration Media Preparation: mTEC Agar

1. Weigh 45.3 g of mTEC agar into a 2 liter Erlenmeyer flask.
2. Add 1.0 liter of deionized water and boil with continuous stirring.
3. Autoclave at 121°C for 15 minutes.
4. Temper the media to 45°C for approximately 30 minutes.
5. Dispense the medium into 9 x 50 mm petri dishes in 4.5 ml aliquots.
6. Store mTEC agar plates inverted at 4°C in the dark for up to one month.

B. Reagent Preparation: Urea/Phenol Red

1. Weigh 2.0 g of urea and 0.01 g of phenol red into a sterile polypropylene bottle.
2. Add 100.0 ml of sterile deionized water and mix well.

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE
ENUMERATION OF FECAL COLIFORMS AND
Escherichia coli IN MARINE WATERS**

ERL-N SOP 1.03.016
REVISION 0
MARCH 1991
PAGE 3 OF 5

C. Diluent Preparation: Phosphate Buffered Saline (PBS)

1. Weigh 8.5 g of sodium chloride into a polypropylene bottle.
2. Add 1.0 liter of deionized water and stir to dissolve.
3. Add 1.25 ml of phosphate buffer and stir well.
4. Autoclave at 121°C for 15 minutes.
5. Store at room temperature for up to one month.

D. Sample Preparation

1. All water samples were shaken vigorously (25 complete up and down movements of 1ft/7sec) prior to transfer of sample to PBS dilution or enrichment tube.

E. Sample Analyses

1. Filter holders are sterilized using the ultraviolet light sterilization unit and inserted into the filter manifold.
2. Filter holders are loaded with membrane filters.
3. Approximately 50.0 ml of sterile PBS is added to the filter holder prior to adding samples of less than or equal to 20.0 ml volumes. Addition of phosphate buffered saline is not required for sample volumes greater than 20.0 ml.
4. An aliquot of sample, or sample dilution, is added to the filter holder.
5. Vacuum is applied to the receiver flask.
6. The walls of the filter holder are rinsed with sterile PBS.
7. Vacuum is applied to draw the rinse buffer through.
8. The filter is removed with sterile forceps and then placed on a mTEC plate.
9. Plates are incubated inverted at 44°C for 20±2 hours.
10. Yellow colonies are scored fecal coliform.
11. Membranes are transferred to pads saturated with urea substrate.
12. After 20 minutes, colonies which maintain their yellow color are scored *Escherichia coli*.

**ERL-N STANDARD OPERATING PROCEDURE
MEMBRANE FILTRATION METHOD FOR THE
ENUMERATION OF FECAL COLIFORMS AND
Escherichia coli IN MARINE WATERS**

ERL-N SOP 1.03.016
REVISION 0
MARCH 1991
PAGE 4 OF 5

IV. TROUBLE SHOOTING

1. Thoroughly rinse the walls of the filter holder prior to removing membrane filters.
2. Sterilize the membrane forceps between samples and dilutions of samples to avoid contamination.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data.
2. Data are used in conjunction with other indicator tests to determine sanitary quality.

VI. REFERENCES

American Public Health Association, Inc. 1970. Recommended Procedures for the Examination of Sea Water and Shellfish. Washington, DC. p. 1-105.

Dufour, A.P., E.R. Strickland and V.J. Cabelli. 1981. Membrane filter method for enumerating *Escherichia coli*. J. Appl. Environ. Microbiol. 41:1152-1158.

ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF *Clostridium perfringens*
IN MARINE WATERS

ERL-N SOP 1.03.017
REVISION 0
MARCH 1991
PAGE 1 OF 4

POINT OF CONTACT:

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I. OBJECTIVE

Clostridium perfringens is often found in the intestines of humans and animals as part of the normal microbiota and is therefore widely distributed in soil and water. This spore-forming microorganism is capable of surviving for long periods of time in the environment, and thus is often used in the detection of fecal contamination that is several years old. Clostridium perfringens is most often encountered in gangrene, but is also known to produce a common type of food poisoning as well as a more serious necrotizing infection of the small bowel. Considering this pathogenicity for man, enumeration of Clostridium perfringens spores in marine waters and shellfish using the most probable number method is often performed as a means of determining the sanitary quality of marine environments.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware

- Blender*
- 1 liter polypropylene bottles
- 20 x 150 mm test tubes
- 10 ml sterile pipettes

*Sterilized at 121°C for 15 minute

B. Media and Diluent

- Media Ingredients:
 - Homogenized milk
 - Iron filings (Klinger-fine grain)
- Diluent Ingredients:
 - Sodium chloride (Baker Analyzed)
 - Phosphate buffer (Baker Analyzed)
 - Deionized water

C. Equipment

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF *Clostridium perfringens*
IN MARINE WATERS**

ERL-N SOP 1.03.017
REVISION 0
MARCH 1991
PAGE 2 OF 4

- Waterbath 45°C
- Analytical balance
- Sterile shucking knives
- Sterile scrubbing brush

III. METHODS

A. Media Preparation: Iron Milk Media

1. Weigh 0.2 g of iron filings into each of fifteen test tubes.
2. Add 10.0 ml of homogenized milk to each test tube.
3. Autoclave at 116°C for 10 minutes.
4. Temper the media to 45°C prior to assay.

B. Diluent Preparation: Phosphate Buffered Saline (PBS)

1. Weigh 8.5 g of sodium chloride into a polypropylene bottle.
2. Add 1.0 liter of deionized water and stir to dissolve.
3. Add 1.25 ml of standard phosphate buffer.
4. Autoclave at 121°C for 15 minutes.
5. Store at room temperature for up to one month.

C. Sample Preparation

1. Waters

- a. All water samples were shaken vigorously (25 complete up and down movements of 1ft/7sec) prior to transfer of sample to dilution or enrichment tube.

2. Shellfish

- a. The hands of the examiner are thoroughly scrubbed with antimicrobial soap and water.
- b. All loose material and growth is scrubbed from the shell using a sterile brush. Particular attention is paid to the crevices at the junctions of the shells.
- c. Entry into the hard clam, *Mercenaria mercenaria*, or into the mussel, *Mytilus* sp., is done using a sterile knife. Never enter through the hinge region.

ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF *Clostridium perfringens*
IN MARINE WATERS

ERL-N SOP 1.03.017
REVISION 0
MARCH 1991
PAGE 3 OF 4

- d. Liquors and meats are transferred to a sterile blender.
- e. Shellfish meats and liquors are blended at 14,000 RPM for 2 minutes.
- f. Homogenized shellfish is stored on ice until assay.

D. Sample Analyses

- 1. Each of 5 iron milk tubes are inoculated with 10.0 ml of the prepared sample (i.e. waters or shellfish homogenate).
- 2. Each of 5 iron milk tubes are inoculated with 1.0 ml of the prepared sample.
- 3. Each of 5 iron milk tubes are inoculated with 1.0 ml of a 10^{-1} dilution of the prepared sample.
- 4. In order to avoid indeterminate results (all positive tubes) extensions of the dilutions mentioned are often required.
- 5. Tubes are incubated at 45°C for 18 hours.
- 6. Tubes are examined for the presence of stormy fermentation.
- 7. Positive tubes (those with stormy fermentation) are scored for Clostridium perfringens.

E. Using the Most Probable Number Geometric Series

- 1. Count the number of positive iron milk tubes from each of the three dilutions performed.
- 2. Use the number of positive tubes from each dilution to form a 3 digit code.
- 3. Locate your three digit code on the most probable number geometric series chart.
- 4. Read the MPN number which corresponds to your three digit code. This is your density of Clostridium perfringens per 100 ml or 100 g of sample.
- 5. When further dilutions of the samples are required, multiply the MPN number by the appropriate dilution which begins the series.

For example, 3 of the 10.0 ml dilutions, 2 of the 1.0 ml dilutions, and 1 of the 0.1 ml dilutions of the sample are positive. The three digit code would be 321. The MPN number for a code of 321 is 14. The density of C. perfringens per 100 ml or 100 g of sample is 14. If the dilutions were 0.1, 0.01 and 0.001, the 3 digit code is 321 and the density of C. perfringens per 100 ml or 100 g of sample is 1400.

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF *Clostridium perfringens*
IN MARINE WATERS**

ERL-N SOP 1.03.017
REVISION 0
MARCH 1991
PAGE 4 OF 4

IV. TROUBLE SHOOTING

1. Thoroughly cleanse shellfish to avoid contamination from outer shell debris.
2. Never enter through the hinge region of shellfish.
3. Samples and dilutions of samples must be properly mixed prior to addition to enrichment tubes.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data.
2. Data are used in conjunction with other indicator tests to determine sanitary quality.

VI. REFERENCES

American Public Health Association, Inc. 1970. Recommended Procedures for the Examination of Sea Water and Shellfish. Washington, DC. p. 1-105.

St. John, W.D., J.R. Matches and M.M. Wekell. 1982. Use of Iron Milk Medium for Enumeration of *Clostridium perfringens*. J. Assoc. Off. Anal. Chem. 65:1129-1133.

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF ENTEROCOCCI IN MARINE
WATERS**

ERL-N SOP 1.03.018
REVISION 0
MARCH 1991
PAGE 1 OF 5

POINT OF CONTACT:

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I. OBJECTIVE

Enterococci, including Streptococcus faecalis and Streptococcus faecium, are found in the intestines of humans and other animals, where they behave as commensals of limited pathogenic potential. Enterococci may cause mild gastrointestinal disease or less commonly, infections of the tissues of the intestines. Because these organisms are associated with fecal wastes of man and animals, they can be isolated from contaminated waters containing such waste. The use of a selective and differential mediums allows for the enumeration of this fecal pollution indicator in marine waters and shellfish using the most probable number method.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware

- Blender*
- 2 liter Erlenmeyer flask
- 16 x 150 mm test tubes
- 20 x 150 mm test tubes
- 1 liter polypropylene bottles
- 10 ml sterile pipettes

*Sterilized at 121°C for 15 minutes

B. Media and Diluent

- Enrichment Media Ingredients:

- Azide Dextrose Broth (Difco)
- Deionized water

-Confirmation Media Ingredients:

- mE agar plates (see SOP/Membrane Filtration Method For the Enumeration of Enterococci in Marine Waters)

- Diluent Ingredients:

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF ENTEROCOCCI IN MARINE
WATERS**

**ERL-N SOP 1.03.018
REVISION 0
MARCH 1991
PAGE 2 OF 5**

- Sodium chloride (Baker Analyzed)
- Phosphate buffer (Baker Analyzed)
- Deionized water

C. Equipment

- Heating mantle with magnetic stirrer
- Analytical balance
- Sterile transfer sticks
- Sterile shucking knives
- Sterile scrubbing brush
- Membranes 0.7 micron (Millipore HCW6047S3)
- Air incubator 35°C
- Air incubator 41°C

III. METHODS

A. Enrichment Media Preparation: Single Strength Azide Dextrose Broth

1. Weigh 34.7 g of azide dextrose broth a 2 liter Erlenmeyer flask.
2. Add 1.0 liter of deionized water and boil with continuous stirring.
3. Dispense the medium in 10.0 ml aliquots into 16 x 150 mm test tubes.
4. Autoclave at 116°C for 10 minutes.
5. Store the media at 4°C for up to one month.

B. Enrichment Media Preparation: Double Strength Azide Dextrose Broth

1. Weigh 69.4 g of azide dextrose broth into a 2 liter Erlenmeyer flask.
2. Add 1.0 liter of deionized water and boil with continuous stirring.
3. Dispense the medium in 10.0 ml aliquots into 20 x 150 mm test tubes.
4. Autoclave at 116°C for 10 minutes.
5. Store media at 4°C for up to one month.

C. Diluent Preparation: Phosphate Buffered Saline (PBS)

1. Weigh 8.5 g of sodium chloride into a polypropylene bottle.
2. Add 1.0 liter of deionized water and stir to dissolve.

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF ENTEROCOCCI IN MARINE
WATERS**

ERL-N SOP 1.03.018
REVISION 0
MARCH 1991
PAGE 3 OF 5

3. Add 1.25 ml of standard phosphate buffer and mix well.
4. Autoclave at 121°C for 15 minutes.
5. Store at room temperature for up to one month.

D. Confirmation Media Preparation: mE Agar

- See SOP/Membrane Filtration Method For the Enumeration of Enterococci in Marine Waters

E. Sample Preparation

1. Waters

- a. All water samples were shaken vigorously, 25 complete up and down movements of 1ft/7sec, prior to transfer of sample to dilution or enrichment tube.

2. Shellfish

- a. The hands of the examiner are thoroughly scrubbed with antimicrobial soap and water.
- b. All loose material and growth is scrubbed from the shell using a sterile brush. Particular attention is paid to the crevices at the junctions of the shells.
- c. Entry into the hard clam, Mercenaria mercenaria, or into the mussel, Mytilus sp., is done using a sterile knife. Never enter through the hinge region.
- d. Liquors and meats are transferred to a sterile blender.
- e. Shellfish meats are blended at 14,000 RPM for 2 minutes.
- f. Homogenized shellfish is stored on ice until assay.

F. Sample Analyses

1. Presumptive Test Using Azide Dextrose Broth

- a. Each of 5 double strength azide dextrose broth tubes are inoculated with 10.0 ml of the prepared sample (i.e. waters or shellfish homogenate).
- b. Each of 5 single strength azide dextrose broth tubes are inoculated with 1.0 ml of the prepared sample.
- c. Each of 5 single strength azide dextrose broth tubes are inoculated with 1.0 ml of a 10^{-1} dilution of the prepared sample.

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF ENTEROCOCCI IN MARINE
WATERS**

ERL-N SOP 1.03.018
REVISION 0
MARCH 1991
PAGE 4 OF 5

- d. In order to avoid indeterminate results, extensions of the dilutions mentioned are often required.
- e. Tubes are incubated at 35°C for 24±2 hours.
- f. Tubes are examined for the presence of growth seen as turbidity in the medium.
- g. Positive turbid tubes are scored.
- h. Confirmation tests are performed at this time for turbid tubes only.
- i. Tubes are returned to 35°C for an additional 24±2 hours.
- j. At 48 hours, the tubes are re-examined for the presence of growth seen as turbidity.
- k. Those tubes which are now turbid are scored and confirmation tests are performed at this time.
- l. Absence of growth/turbidity at the end of 48 hours constitutes a negative test result for the enterococci group.

2. Confirmation Test Using mE Agar

- a. A sterile 0.75 micron membrane is placed on a mE agar plate.
- b. Sterile transfer sticks are used to transfer culture bacteria from turbid azide dextrose broth tubes to the membrane of the mE agar plate.
- c. Plates are incubated at 41°C for 48 hours.
- d. Plates are examined for the presence of blue colonies, with pink centers and gray fringes, on the membrane.
- e. Colonies possessing this appearance are scored positive for enterococci.

G. Using the Most Probable Number Geometric Series

- 1. Count the number of positive azide dextrose tubes (those with positive confirmations) from each of the three dilutions performed.
- 2. Use the number of positive tubes from each dilution to form a 3 digit code.
- 3. Locate your three digit code on the most probable number geometric series chart.
- 4. Read the MPN number which corresponds to your three digit code. This is your

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF ENTEROCOCCI IN MARINE
WATERS**

ERL-N SOP 1.03.018
REVISION 0
MARCH 1991
PAGE 5 OF 5

density per 100 ml or 100 g of sample.

5. When further dilutions of the samples are required, multiply the MPN number by the appropriate dilution which begins the series.

For example, 3 of the 10 ml dilutions, 2 of the 1 ml dilutions, and 1 of the 0.1 ml dilutions of the sample are positive. The three digit code would be 321. The MPN number for a code of 321 is 14. The density of enterococci per 100 ml or 100 g of sample is 14. If the dilutions were 0.1, 0.01 and 0.001, the 3 digit code is 321 and the density of enterococci per 100 ml or 100 g of sample is 1400.

IV. TROUBLE SHOOTING

1. Thoroughly cleanse shellfish to avoid contamination from outer shell debris.
2. Never enter through the hinge region shellfish.
3. Samples and dilutions of samples must be properly mixed prior to addition to enrichment tubes.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Tabulate and summarize the data.
2. Data are used in conjunction with other indicator tests to determine sanitary quality.

VI. REFERENCES

- American Public Health Association, Inc. 1970. Recommended Procedures for the Examination of Sea Water and Shellfish. Washington, DC. p. 1-105.
- Levin, M.A., S.R. Fischer, V.J. Cabelli. 1975. Membrane Filter Technique for Enumeration of Enterococci in Marine Waters. Appl. Microbiol. 30:66-71.

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF FECAL COLIFORMS AND
Escherichia coli IN MARINE WATERS**

ERL-N SOP 1.03.019
REVISION 0
MARCH 1991
PAGE 1 OF 6

POINT OF CONTACT:

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I. OBJECTIVE

Fecal coliforms, including *Escherichia coli*, are found in the intestinal tract of man and both warm- and cold-blooded animals. For this reason, they are often employed as indicators of fecal contamination. The National Shellfish Sanitation Program (NSSP) requires that a sanitary survey be performed of every potential shellfish growing area. The sanitary survey is a microbiological evaluation of all actual and potential pollution sources and environmental factors which may influence the water quality in shellfish growing areas. Fecal coliform density, as determined by the most probable number method, is the accepted standard for the classification of shellfish growing waters. The NSSP requires that the geometric mean fecal coliform density does not exceed 14 colony forming units per 100 ml of sample and that no more than 10 percent of the samples exceed a density of 43 colony forming units per 100 ml of sample. Enumeration of fecal coliforms and *Escherichia coli* in marine waters and shellfish using the most probable method is a means of determining the sanitary quality of marine environments.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware

- Blender*
- 2 liter Erlenmeyer flask
- 6 x 50 mm culture tubes
- 16 x 100 mm test tubes
- 16 x 150 mm test tubes
- 20 x 150 mm test tubes
- 1 liter polypropylene bottles
- 10 ml sterile pipettes

*Sterilized at 121°C for 15 minute

B. Media and Diluent

- Enrichment Media Ingredients:
 - Lauryl Tryptose Broth (Difco)
 - Deionized water

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF FECAL COLIFORMS AND
Escherichia coli IN MARINE WATERS**

ERL-N SOP 1.03.019
REVISION 0
MARCH 1991
PAGE 2 OF 6

-Confirmation Media Ingredients:

- EC Medium (Difco)
- 4-Methylumbelliferyl-B-D-Glucuronide/MUG (Sigma)

- Diluent Ingredients:

- Sodium chloride (Baker Analyzed)
- Phosphate buffer (Baker Analyzed)
- Deionized water

C. Equipment

- Heating mantle with magnetic stirrer
- Analytical balance
- Sterile transfer sticks
- Sterile shucking knives
- Sterile scrubbing brush
- Air incubator 35°C
- Water bath 44.5°C
- Black ray lamp (UVL-21, UV-366NM, UVP, Inc.)

III. METHODS

A. Enrichment Media Preparation: Single Strength Lauryl Tryptose Broth

1. Weigh 35.6 g of lauryl tryptose broth a 2 liter Erlenmeyer flask.
2. Add 1.0 liter of deionized water and boil with continuous stirring.
3. Dispense the medium in 10.0 ml aliquots into 16 x 150 mm test tubes containing inverted 6 x 50 mm culture tubes.
4. Autoclave at 121°C for 15 minutes.
5. Store the media at 4°C for up to one month.

B. Enrichment Media Preparation: Double Strength Lauryl Tryptose Broth

1. Weigh 71.2 g of lauryl tryptose broth into a 2 liter Erlenmyer flask.
2. Add 1.0 liter of deionized water and boil with continuous stirring.
3. Dispense the medium in 10.0 ml aliquots into 20 x 150 mm test tubes containing inverted 6 x 50 mm culture tubes.

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF FECAL COLIFORMS AND
Escherichia coli IN MARINE WATERS**

ERL-N SOP 1.03.019
REVISION 0
MARCH 1991
PAGE 3 OF 6

4. Autoclave at 121°C for 15 minutes.
5. Store media at 4°C for up to one month.

C. Confirmation Media Preparation: EC Mug Medium

1. Weigh 37.0 g of EC medium and 0.05 g of 4-methylumbelliferyl-B-D-glucuronide into a 2 liter Erlenmeyer flask.
2. Add 1.0 liter of deionized water and boil with continuous stirring.
3. Dispense the medium in 5.0 ml aliquots into 16 x 100 mm test tubes containing inverted 6 x 50 mm culture tubes.
4. Autoclave at 121°C for 15 minutes.
5. Store media at 4°C in the dark for up to one month.

D. Diluent Preparation: Phosphate Buffered Saline (PBS)

1. Weigh 8.5 g of sodium chloride into a polypropylene bottle.
2. Add 1.0 liter of deionized water and stir to dissolve.
3. Add 1.25 ml of standard phosphate buffer.
4. Autoclave at 121°C for 15 minutes.
5. Store at room temperature for up to one month.

E. Sample Preparation

1. Waters

- a. All water samples were shaken vigorously (25 complete up and down movements of 1ft/7sec) prior to transfer of sample to dilution or enrichment tube.

2. Shellfish

- a. The hands of the examiner are thoroughly scrubbed with antimicrobial soap and water.
- b. All loose material and growth is scrubbed from the shell using a sterile brush. Particular attention is paid to the crevices at the junctions of the shells.

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF FECAL COLIFORMS AND
Escherichia coli IN MARINE WATERS**

ERL-N SOP 1.03.019
REVISION 0
MARCH 1991
PAGE 4 OF 6

- c. Entry into the hard clam, *Mercenaria mercenaria*, or into the mussel, *Mytilus* sp., is done using a sterile knife. Never enter through the hinge region.
- d. Liquors and meats are transferred to a sterile blender.
- e. Shellfish meats and liquors are blended at 14,000 RPM for 2 minutes.
- f. Homogenized shellfish is stored on ice until assay.

F. Sample Analyses

1. Presumptive Test Using Lauryl Tryptose Broth

- a. Each of 5 double strength lauryl tryptose broth tubes are inoculated with 10.0 ml of the prepared sample (i.e. waters or shellfish homogenate).
- b. Each of 5 single strength lauryl tryptose broth tubes are inoculated with 1.0 ml of the prepared sample.
- c. Each of 5 single strength lauryl tryptose broth tubes are inoculated with 1.0 ml of a 10^{-1} dilution of the prepared sample.
- d. In order to avoid indeterminate results, extensions of the dilutions mentioned are often required.
- e. Tubes are incubated at 35°C for 24±2 hours.
- f. Tubes are examined for the presence/absence of gas production.
- g. Tubes with gas production are scored and confirmation tests are performed (for only those tubes with gas production).
- h. Tubes are returned to 35°C for an additional 24±2 hours.
- i. At 48 hours, the tubes are re-examined for the presence/absence of gas production.
- j. Those tubes which have gas production are scored, and confirmation tests are performed at this time.
- k. Absence of gas production at the end of 48 hours constitutes a negative test result for the fecal coliform group.

2. Confirmation Test Using EC-MUG Medium

- a. Sterile transfer sticks are used to transfer bacteria from lauryl tryptose broth tubes with gas production to EC-MUG tubes.

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF FECAL COLIFORMS AND
Escherichia coli IN MARINE WATERS**

ERL-N SOP 1.03.019
REVISION 0
MARCH 1991
PAGE 5 OF 6

- b. Tubes are incubated at 44.5°C for 24±2 hours.
- c. Tubes are examined for the presence/absence of gas production as well as fluorescence using a black ray lamp held approximately 1 foot from the tubes.
- d. Tubes with gas production and fluorescence are scored *Escherichia coli*.
- e. Tubes with only gas production are scored fecal coliform.

G. Using the Most Probable Number Geometric Series

- 1. Count the number of positive tubes from each of the three dilutions performed.
- 2. Use the number of positive tubes from each dilution to form a 3 digit code.
- 3. Locate your three digit code on the most probable number geometric series chart.
- 4. Read the MPN number which corresponds to your three digit code. This is your density per 100 ml of sample.
- 5. When further dilutions of the samples are required, multiply the MPN number by the appropriate dilution which begins the series.

For example, 3 of the 10 ml dilutions, 2 of the 1 ml dilutions, and 1 of the 0.1 ml dilutions of the sample are positive. The three digit code would be 321. The MPN number for a code of 321 is 14. The density of enterococci per 100 ml of sample is 14. If the dilutions were 0.1, 0.01 and 0.001, the 3 digit code is 321 and the density of enterococci per 100 ml is 1400.

IV. TROUBLE SHOOTING

- 1. Thoroughly cleanse shellfish to avoid contamination from outer shell debris.
- 2. Never enter through the hinge region of shellfish.
- 3. Samples and dilutions of samples must be properly mixed prior to addition to enrichment tubes.

V. STATISTICAL ANALYSIS AND DATA USAGE

- 1. Tabulate and summarize the data.
- 2. Data are used in conjunction with other indicator tests to determine sanitary quality.

**ERL-N STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF FECAL COLIFORMS AND
Escherichia coli IN MARINE WATERS**

ERL-N SOP 1.03.019
REVISION 0
MARCH 1991
PAGE 6 OF 6

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**ERL-N STANDARD OPERATING PROCEDURE
MEASUREMENT OF THE ECOLOGICAL EFFECTS,
FATE, AND TRANSPORT OF CHEMICALS IN A
SITE-SPECIFIC EXPERIMENTAL MARINE MICROCOSM**

ERL-N SOP 1.03.020
REVISION 0
DECEMBER 1990
PAGE 1 OF 21

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I. OBJECTIVE

The objective of this protocol is to determine, through the use of a marine microcosm, the ecological effects of a photo-stable chemical substance or mixture and the potential for indirect human exposures through the consumption of marine organisms. Undisturbed, natural pelagic and benthic communities are coupled within a single system whose physical and chemical conditions simulate those in the natural system in order to generate data that are far more applicable to predictive environmental and ecological assessments than those derived from more simple tests. Furthermore, since the information generated via microcosm experiments is integrated at the system level of organization, quantitative relationships can be determined for factors such as chemical exposure concentrations and transport rates to measured body burdens, ecological effects and input concentrations/loadings of the test substance.

II. NECESSARY MATERIALS AND EQUIPMENT

Included in Methods Section.

III. METHODS

1. DEFINITION OF A NATURAL SYSTEM

1.1 The natural marine system (designated as "natural system") at a particular geographic location consists of an air-water interface and a coupled water column and benthic subsystem. Within both the water column and benthic subsystems certain physical and chemical conditions, and biological properties, exist. These conditions and properties are simulated in laboratory microcosms as described in Section 3.1.2 and 4.6.2.

1.1.1 The physical and chemical conditions of the natural system are defined in terms of its:

- (1) **Boundaries** - The boundaries of the natural system are delineated by the maximum horizontal tidal excursion of water around a selected point.
- (2) **Light** - The light regime of the natural system is characterized by: the

**ERL-N STANDARD OPERATING PROCEDURE
MEASUREMENT OF THE ECOLOGICAL EFFECTS,
FATE, AND TRANSPORT OF CHEMICALS IN A
SITE-SPECIFIC EXPERIMENTAL MARINE MICROCOSM**

ERL-N SOP 1.03.020
REVISION 0
DECEMBER 1990
PAGE 2 OF 21

photoperiod, the best estimate of average monthly incident radiation (total incident radiation per day divided by photoperiod averaged over one month), the average seasonal water extinction coefficient, and the depth at which the average light intensity occurs.

- (3) Surface microlayer - That portion of the air-water interface which can be sampled for critical substances and biota by various types of membrane filters e.g., cellulose acetate, polycarbonate, etc., placed on the water surface and then immediately removed.
- (4) Sea water - That fluid existing within the boundaries of the natural system.
- (5) Sea water turbulence - The average water motion realized for the entire water column of the natural system during the period of experimentation.
- (6) Sea water turnover rate - The time required for one complete water turnover or exchange within the defined boundaries of the natural system.
- (7) Ratio of benthic surface area to sea water volume - This ratio is obtained by dividing the calculated benthic surface area of the natural system by the best estimate of water volume of the system.
- (8) Sediment - The bottom substrate existing, at the mean water depth, within the boundaries of the natural system. Sediments within this area containing ecologically important communities, or communities which are important in the [human] food web should be used.
- (9) Sea water flow rate over sediment surfaces - The average tidal velocity over the bottom as measured in the natural system.

1.2 Biological Conditions and Descriptions

1.2.1 The biota of the natural system is characterized by the organisms in the surface microlayer, water column and benthic subsystem.

- (1) The surface microlayer community is characterized by the type and number of microbiota at the air-water interface. This community structure is determined by placing a membrane on the water surface of the microcosm. The type and number of microbiota found on the membrane, relative to subsurface microbiota, defines this community.
- (2) The water column community is characterized by the numbers and species composition of phytoplankton, zooplankton, and transient larval forms found in the sea water at the designated collection site at mid-tide. The water column, with its assemblages of biota, is obtained from the natural system by the methods described for filling the microcosms (Section 4.6.2(1)).
- (3) The benthic subsystem is characterized by the sediment type and the structural

**ERL-N STANDARD OPERATING PROCEDURE
MEASUREMENT OF THE ECOLOGICAL EFFECTS,
FATE, AND TRANSPORT OF CHEMICALS IN A
SITE-SPECIFIC EXPERIMENTAL MARINE MICROCOSM**

ERL-N SOP 1.03.020
REVISION 0
DECEMBER 1990
PAGE 3 OF 21

composition of the benthic community, i.e., numbers, species composition, and feeding types of organisms collected from the benthic boxes (Section 3.1.2.1(2 & 3)) and retained on a 0.5 mm screen.

- (4) If the natural system has more than one distinct benthic community, those that have direct routes to human consumption and/or are of significant economic and aesthetic importance should be considered for experimental use.

2. TEST SUBSTANCE

2.1 Information Required

- 2.1.1 A detailed description of the test substances (radiolabeled and unlabeled) including its source, lot number, composition and physical chemical properties must be provided. It is recommended that the test substance be radiolabeled to enable the rapid, accurate measurement of the original test material and potential transformation products, both within and outside the microcosm. Other applicable analytical techniques (Section 4.6.4(8)) should also be utilized.
- 2.1.2 The degree of purity and the identification and quantification of any impurities must be established.
- 2.1.3 It is extremely important to have a knowledge of, or a best estimate of, the test substance's mode and form of entry into the marine environment. Specifically, does the test substance enter by atmospheric or aqueous sources? Is the test substance in the form of a gas, liquid, or solid? Is the test substance in association with other materials such as a carrier or solvent mixture? As will be discussed in Section 4.5.1, the extent of such information will determine the method by which the test substance is added to the experimental microcosms. In addition, the test substance's photo-oxidation potential, solubility in sea water (within the salinity and temperature range of the natural system), solubility in potential carriers (if appropriate as per Section 4.5.1 (3)), vapor pressure, specific gravity (if a liquid), and octanol:water partition coefficient should be established.

2.2 Methodology

- 2.2.1 The investigator must provide descriptions of methods for collecting and measuring the test substance and potential transformation products in and/or on the: gas phase, surface microlayer (if the test substance partitions to the air-water interface), sea water (both particulate, $\geq 0.22 \mu\text{m}$, and dissolved, $< 0.22 \mu\text{m}$), sediment, biota (both whole animal and specific tissues if necessary), and microcosm (glass) surfaces.

3. MICROCOSM FACILITY

- 3.1 The microcosm facility (Figure 1) is designed to simulate relevant physical, chemical, and biological conditions existing in the natural system described in Section 2. It is composed of two basic parts: (1) the support equipment (room, waterbath, light and turbulence fixtures, test compartments, and an air evacuation system) and (2) the

**ERL-N STANDARD OPERATING PROCEDURE
MEASUREMENT OF THE ECOLOGICAL EFFECTS,
FATE, AND TRANSPORT OF CHEMICALS IN A
SITE-SPECIFIC EXPERIMENTAL MARINE MICROCOSM**

ERL-N SOP 1.03.020
REVISION 0
DECEMBER 1990
PAGE 4 OF 21

experimental microcosms (tanks, paddles, benthic cylinders, pumps and pump air supply).

3.1.1 Support Equipment (See Figure 1)

- (1) All testing must be performed in a room that eliminates any light source other than that provided by the microcosm facility.
- (2) The microcosm tanks must be held in a waterbath of sufficient dimensions and flow rate as to provide uniform test temperatures within all treatment conditions and to maintain those temperatures within 1°C of the natural system.
- (3) A canopy above the waterbath should hold sufficient fluorescent lighting to provide an even light distribution of $50\mu\text{E}/\text{m}^2/\text{sec}$ over all the experimental microcosms. Light is provided by "Cool White" fluorescent bulbs, and the photoperiod (sunrise to sunset) controlled by a 24 hour timer.
- (4) Turbulence is provided by rotating glass "paddles" driven by a system that consists of an electric motor, chain drive, mounts and drive shafts. This system is mounted on the canopy, through which the drive shafts extend, directly above each microcosm tank (Figure 1). The paddles are connected to the drive shafts with nylon bolts. The speed and direction of the paddles are controllable, with the direction being automatically reversed at regular intervals (i.e. 30 seconds) by an electronic controller (Figure 2) to maximize turbulence.
- (5) The open area between the canopy and waterbath is enclosed and sealed with plexiglass. The front plexiglass covers are removable to facilitate setting up and filling the microcosm tanks. Plexiglass panels are also mounted transversely within the waterbath/canopy module, extending approximately five cm below the water surface providing relatively airtight test compartments. The number of test compartments is dependent upon the number of treatments. All tanks within a given test compartment must have the same concentration of the test substance. Access to the microcosm tanks during an experiment is via hinged ports mounted in the front plexiglass cover.
- (6) A slightly negative air pressure is maintained within each test compartment. An exhaust fan draws air from the compartments through a PVC pipe manifold. The exhaust air is passed through activated charcoal filters and vented through a stack outside of the laboratory building (see Figure 1).

3.1.2 Experimental Microcosms

- 3.1.2.1** The experimental microcosms are designed to provide a multi-trophic level experimental chamber that has coupled pelagic (water column) and benthic communities similar to those existing in the natural system.

- (1) The microcosm tanks are 160 liter, cylindrical, hard glass containers. The tanks are held in position by a plywood "collar" mounted in the water bath trough and encircling each tank approximately 15 cm below the rim.
- (2) The benthic cylinders are thick walled, approximately 30 cm tall, hard glass cylinders which are open at both ends (Figure 3) with a small (ca. 1.5 cm) overflow port located at a height of ca. 25 cm from the bottom. The inner diameter of the benthic cylinder must be such that the microcosm's sediment surface area to water volume ratio equals that of the natural system (Section 1.1.1(7)).
- (3) The benthic cylinders are used to collect undisturbed sediment cores from the prescribed portion of the natural system (1.1.1(8) and 1.2.1(4)). Cores are obtained in situ by divers or a non-disruptive coring device. The depths of the cores should be equal to or greater than the depth of biological habitation or activity but not to exceed 25 cm. The bottoms of the cylinders (cores) are sealed, prior to installation in the microcosm tanks, by placing them in slightly larger diameter glass containers (designated as benthic core containers; Figure 3). The height of these containers should be to just below the level of the overflow port. This creates equal hydrostatic pressures inside and outside the chamber. The cores, at this point, should be watertight i.e., the water overlying the sediment core will not flow downward between the sediment periphery and the benthic cylinder. A right angle glass "drip" tube is inserted into the overflow port of the benthic cylinder. The tube is long enough to assure that water exiting the core will enter the microcosm directly rather than drip down the side of the benthic core (see Figure 3). Cores should be placed in the microcosm on the same day in which they are collected. During storage at sea and transport back to the laboratory, cores should be submerged in flowing sea water. The flow rate must be slow enough as to be non-disruptive to the sediment surface.
- (4) Water flow over the sediment core in the microcosm is provided by an all glass, air displacement pump (Figure 3). The dimensions of the pump should be sufficient to provide a flow comparable to the average bottom water flow rate of the natural system. The pump is positioned in the tank adjacent to the benthic core (Figures 1 & 3). The benthic core and associated glassware is mounted in the microcosm tank so that the cylinder overflow port is approximately five cm above the tank water level. The cylinder and pump are supported by a glass rod platform that attaches to the rim of the tank (Figure 3). Water from the microcosm tank enters the displacement pump through a small (2 cm) hole in the bottom. When air pressure is applied to the pump a thin glass coverslip inside the pump is forced downward and seals the bottom hole. This forces water in the pump through a connecting glass tube and into the benthic cylinder. To minimize sediment disturbance and resuspension, the water entering the benthic cylinder is directed into several horizontal streams by a glass diffuser three to five cm above the sediment surface (Figure 3). Water overflows from the benthic cylinder back into the microcosm tank

through the overflow port and drip tube located approximately five cm above the microcosm water surface (10 cm above the sediment surface: Figure 3). An electronic timer controls the on/off cycle of the benthic pump via control valves in the compressed air lines (Figure 4). When the air flow is stopped and vented, water re-enters the pump from the microcosm tank. The timer is adjusted to set the water flow rate to approximate the mean tidal velocity expressed at the sediment surface of the natural system (Section 1.1.1(9)).

- (5) The light intensity over the sediment surface should approximate that incident in the bottom meter of the natural system. This is accomplished by placing a light filter or cover over the top of the benthic cylinder (Figure 3). See Section 4.5.3 for establishment of water column light intensity.
- (6) Turbulence is generated by glass paddles that are connected to the drive shafts described in Section 3.1.1(4) and Figures 1 & 2. The paddle consists of a vertical shaft with four to five rows of smaller diameter glass shafts fused to it radiating horizontally outward (Figure 2). The length and number of arms are dependent on the microcosm tank, benthic cylinder and pump configurations, and the level of turbulence desired. The turbulence level may also be controlled by regulating the speed of rotation. The motor controller (Figure 2) automatically reverses the direction of rotation every 30 seconds to create a more uniform field of water turbulence. The speed of paddle rotation is adjusted so that the dissolution rate (described as weight loss/time) of pure gypsum (CaSO_4) blocks (1.5 cm x 2.5 cm x 1.0 cm), distributed throughout a microcosm tank is statistically equivalent to the dissolution rates of gypsum blocks measured in the natural system during the season of the experiment. Dissolution rates should be measured and water turbulence adjusted prior to each experiment.
- (7) If the test substance forms a surface film that could contaminate water samples as they are removed from the microcosm, then a small "surface film protector" must be installed in order to allow collection of non-contaminated sub-surface samples. The protector consists of a glass cylinder (4.5 cm diam. x 6 cm long) that is suspended from the side of the tank and extends several centimeters above and below the surface of the water (Figure 5). Any surface film within the cylinder can be temporarily removed by wicking with an absorbent/adsorbent material, e.g., cellulose acetate filter pad. Water samples can then be taken through the cylinder with minimal surface film contamination.
- (8) All test equipment and sampling devices that come in contact with sea water and test solutions must be pre-treated by washing with detergent, rinsing with deionized water, and heating in a muffle furnace to 550°C for at least 30 minutes prior to use.

4. TEST PROCEDURES

**ERL-N STANDARD OPERATING PROCEDURE
MEASUREMENT OF THE ECOLOGICAL EFFECTS,
FATE, AND TRANSPORT OF CHEMICALS IN A
SITE-SPECIFIC EXPERIMENTAL MARINE MICROCOSM**

ERL-N SOP 1.03.020
REVISION 0
DECEMBER 1990
PAGE 7 OF 21

4.1 Assumptions

4.1.1 The validity of extrapolation of perturbation or test substance results derived from the experimental microcosms to the natural system is based on how well unperturbed biological events in the experimental control tanks mimic those that occur in the natural system at the time of the experiment. For those biological events that are statistically equivalent ($\alpha = 0.05$), it is assumed that perturbation behavior observed in the experimental tanks would also be realized in the natural system if it was similarly perturbed.

4.1.2 As per section 4.3.1 (1), it is assumed that the arbitrarily chosen mode and form of entry of the test substance into the microcosms will be sufficiently equal to the actual entry into the natural system to produce equivalent results in a similarly perturbed natural system.

4.2 Test of Assumption 4.1.1

4.2.1 Chemical fate and ecological effects of the test substance can be ascertained in the experimental microcosms and related to the expected perturbation behavior in a natural system provided the levels of all relevant independent variables, either simulated and/or realized in the microcosms, are equal to those in the natural system at the time of the test.

4.3 Preliminary Testing

4.3.1 Input of test substance: mode, form and exposure levels.

- (1) If the mode (i.e., atmospheric and/or aqueous) and form (i.e., gas, liquid or solid) of entry of the test substance into the marine environment is known (documentation required), then similar mechanisms of entry should be simulated in the experimental microcosms. If the only mode of entry is the atmosphere, then this protocol is not applicable at this time. However, if the investigators can create a realistic input of the test substance through the gas phase, then such a mode of entry is acceptable. If the mode of entry is partially or completely aqueous, then the test substance, in its realistic form, is added to achieve appropriate nominal water concentrations based on field-measured concentrations. If such field data are not available, concentrations of 1, 10, and 100 $\mu\text{g/l}$ are recommended.
- (2) If the mode and form of entry of the test substance into the marine environment is unknown, then the mode of entry into the microcosms should be aqueous.
- (3) The form of entry will depend upon the solubility of the test substance in sea water. If the test substance is only sparingly soluble in sea water, then an "appropriate" carrier will have to be used. An appropriate carrier would (a) completely dissolve the test substance and (b) result in a uniform water column distribution of the test substance at the start of the experiment (see

definitive test, Section 4.4). An "inappropriate" carrier would cause the test substance to be distributed within one compartment of the microcosm in a short period of time. For example, using pentane as the carrier would result in the majority of the test substance being distributed at the surface of the water column at the start of the experiment. With methylene chloride, the test substance would be found on the bottom of the microcosm tank and subsequently removed during microcosm cleaning (see Section 4.4.1(4)). Acetone is an appropriate carrier and is recommended where applicable. Use of an appropriate carrier results in the desired homogeneous water column concentrations of the test substance at the start of the experiment.

4.3.2 Sampling and measurement

Preliminary physical and chemical experiments should be performed to determine appropriate measurement techniques and the most efficient sampling program. These tests should develop information regarding methods for capturing and measuring the test substance and potential transformation products in the water, surface microlayer (if a surface film is formed), gas, sediment, tissues, and on glass surfaces.

4.3.3 Light Intensity

Irradiance is set at $50\mu\text{E}/\text{m}^2/\text{sec}$ or greater for the duration of the test, with seasonal photoperiods being simulated. Aluminum foil should be placed over the fluorescent light where needed in order to achieve uniform irradiance across the entire microcosm surface (this needs to be tested).

4.3.4 Cleaning Schedule

A cleaning schedule must be determined which will prevent the buildup of fouling organisms on exposed glass surfaces. Measured phytoplankton concentrations are used to determine the proper cleaning schedule. Several different cleaning frequencies are employed and plotted against phytoplankton numbers. The frequency at which the microcosm plankton community is most similar to that of the natural system represents the desired cleaning frequency. This must be determined for the season in which testing will be performed. Based on studies in Narragansett Bay, RI, cleaning the wall surfaces every other day was sufficient to simulate realistic phytoplankton densities within the experimental microcosms.

4.4 Definitive Test

4.4.1 The definitive test will consist of dosing the microcosms with three concentrations of the test substance as established in Section 4.3.1(1). Each concentration should have at least five replicates. Any manipulation (i.e., sampling, turning off the pumps or paddles, etc.) that is performed on one tank must be performed on all tanks.

- (1) There should be at least five replicate control microcosms. If a carrier is used (Section 4.3.1 (3)), then five microcosms with just carrier added (carrier controls) and five sea water (carrier-free) controls should be used.

- (2) The definitive test will consist of:
 - (a) Addition of the test substance to the water column of the experimental microcosms at the start of the test.
 - (b) Performance of the test for 30 days, during which the continued addition of the test substance to the microcosm is consistent with the release pattern expected for introduction of the substance into the marine environment, or is performed immediately following a water exchange in sufficient volume to bring only the replacement water up to the initial concentration. It is recommended that definitive tests be conducted during the temperature extremes of an annual cycle, i.e., both winter and summer.
- (3) During the 30 day exposure period, one tank from each treatment condition may be sacrificed on days 10 and 20 and subjected to comprehensive sampling (Section 4.4.5) to determine the temporal dynamics of bioaccumulation and sediment accumulation of the test substance. Following the exposure period (day 30), all remaining microcosms should be similarly sacrificed and sampled (Section 4.4.6).
- (4) To clean the walls and other surfaces of the microcosms, the entire water body of a single microcosm is removed by connecting it to a large, centrally-located glass vessel and then applying negative pressure to this holding chamber. This transfers the water to this chamber where it is stored during the cleaning process. This vessel will have entry ports for sea water, wet vacuum, positive air pressure, and deionized or solvent washes located at the top of the vessel. Trough (water bath) water should be removed prior to removing microcosm water to prevent damage to the tank, as the microcosm tank will become buoyant as the water is removed. Following removal of the microcosm water, all glass surfaces are sprayed with hot (71°C) deionized water. Surfaces washed include the tank walls, benthic pumps, glass stirring paddles, pump and benthic container supports and the outside of the benthic chamber. All washing is performed in the empty tank. Cleaning water and residue are then removed into a small (20 l) vacuum flask and saved for chemical analysis. The inside of the benthic cylinder is cleaned by carefully wiping the wall above the sediment surface with a Kimwipe™ or other absorbent material. Following this procedure, the water from the glass holding vessel is returned to the microcosm, using positive air pressure. After last replicate tank within each concentration level is cleaned, the inside of the glass holding vessel is then rinsed with hot DI (or a solvent that kills fouling organisms and is miscible with the test substance) and this water (or solvent) saved for analysis (Section 4.4.4(8)). Cleaning should commence with the controls and end with the highest concentration tested. At the end of a cleaning cycle, it is important to remove, using DI or solvents, all traces of the test substance from the glass holding vessel. This procedure will eliminate contamination of the control and lower concentration replicates during the next cleaning cycle. A cleaning schedule must be determined for

each season during which the experiment will be conducted (see Section 4.3.4). Where possible, cleaning should be scheduled to coincide with sea water renewals.

- (5) When radiolabeled compounds are used, a radioactivity budget is calculated for each microcosm (see section V, 2.2.1). Radioactivity in the water is measured both before and after each cleaning cycle. If the drop in radioactivity exceeds two percent, the amount of radiolabelled test substance lost is replaced.

4.4.2 The microcosm test system is placed in operation as follows:

- (1) The test water is collected from the natural system at mid-tide by hand bucketing or non-destructive pumping (vacuum), and transported to the test facility in glass tanks. If the natural system is vertically stratified in terms of temperature and/or salinity, the initial filling water should be a composite of water collected from various depths. Subsequent collections for water exchange (Section 4.4.2(8)) and biological sampling (Section 4.4.5) are performed in the same manner.
- (2) All microcosm tanks are filled 24 hours prior to the installation of benthic cores. During the filling process, equal amounts of water (e.g., 10 L) are sequentially added to each tank. This process is repeated until all tanks are filled to the prescribed volume. This insures equal water column communities among all microcosms.
- (3) The paddles are installed and the speed of rotation is established (see Section 3.1.2.1(6)) to generate a turbulence level equivalent to that in the natural system.
- (4) Irradiance at the microcosm tank water surface is controlled by adjusting the shading of fluorescent lights to achieve levels as determined in Section 4.3.3. Photoperiod is set equal to ambient conditions.
- (5) Water flow in the water bath is adjusted to provide temperature tracking within 1°C of the natural system. Flow rates must also be sufficient to maintain all experimental tanks within 1°C of each other.
- (6) The benthic cylinders and glass containers (Section 3.1.2.1(2 & 3)) are placed in the tanks (Section 3.1.2.1(4)) and any disturbed sediment allowed to settle (approximately 10 minutes).
- (7) The benthic pumps are placed adjacent to and connected to the benthic cores (Figure 3) and air lines (Figure 1) supplying low pressure air (5-6 psi) are attached. After the disturbed sediment in the benthic cylinders settles, the low pressure air to the benthic pumps is turned on and water flow to the benthic cylinders begun at an average rate equal to the average tidal flow over the benthic substrate in the natural system (Section 3.1.2.1(4)).

- (8) Water turnover volume is established to match the exchange rate of the natural system (Section 1.1.1(6)). The frequency of water exchange in the microcosm tanks should be at least three times per week, and should coincide with cleaning and the biological and chemical sample collections. The discrete volume of water removed during water exchange equals the calculated amount minus that volume removed during biological and chemical sampling. Water removed is saved for chemical and biological analyses and for determination of settleable particulates (Section 4.4.5(6)).
- (9) If a surface film of the test substance is formed, a portion of that film must be removed during each water exchange to simulate natural advective transport. The area of surface film to be removed by wicking with filter pads or other suitably absorbent material (Section 3.1.2.1(7)) is defined by:

$$\text{Area (cm)} = \frac{\text{daily water turnover volume}}{\text{total microcosm water volume}} \times \text{benthic surface area}$$

4.4.3 Test Substance Addition

4.4.3.1 Carriers

- (1) If a carrier other than sea water is used to dissolve the test substance (Section 4.3.1), the volume used should not exceed the minimum necessary to dissolve and/or create a homogeneous suspension of the test substance in the microcosm. If the test substance is a mixture, formulation or a commercial product, none of the ingredients is considered a carrier unless an extra amount is used to prepare the stock solution.
- (2) Acetone is the recommended carrier. After addition to the microcosm, the water column concentration of acetone should not exceed 10 mg/l.
- (3) If the test substance is not soluble in acetone, a solvent that has the least ecological effects on the microcosm test system should be used.

4.4.3.2 Addition to Test Container

- (1) The test substance and carrier, if necessary as per Section 4.3.1, should be added to the microcosm in such a manner as to insure that the initial substance distribution is homogenous in the water column.
- (2) The test substance must be added to the microcosm tanks in the same manner as the initial addition whenever there is a water exchange (Section 4.4.2(8)) but only in quantities sufficient to achieve the desired test concentrations in the replacement water.

**ERL-N STANDARD OPERATING PROCEDURE
MEASUREMENT OF THE ECOLOGICAL EFFECTS,
FATE, AND TRANSPORT OF CHEMICALS IN A
SITE-SPECIFIC EXPERIMENTAL MARINE MICROCOSM**

**ERL-N SOP 1.03.020
REVISION 0
DECEMBER 1990
PAGE 12 OF 21**

- (3) The addition of a carrier to the selected carrier control tanks (Section 4.4.1(1)) must be performed in the same manner and at the same volume and frequency as is done in the exposed tanks.

4.4.4 Chemical Fate Sampling

- (1) Water samples to determine test substance levels in the water column should be taken five minutes after the initial addition and then at 1, 3, 6, 12 and 24 hours. Thereafter, these samples should be taken prior to each water exchange. If the test substance is radiolabelled, then sample volumes of 10 ml should be adequate to separate parent from breakdown products. Water samples are filtered through a 0.22 μ m filter and both the dissolved and particulate phases analyzed (Section 4.4.4(8)).
- (2) Water samples for chemical analysis must be collected: (a) While the turbulence (paddle) system is operating. (b) At a point away from the side of the tank and at least 2 cm below the air-water interface.
- (3) Representative samples of the various zooplankton species should be collected once a week (see Section 4.4.5(4)) to analyze for uptake and accumulation of the test substance and, if possible, transformation products.
- (4) If a surface film is formed by the test substance, it should be sampled using an absorbent filter pad (Section 3.1.2.1(7)) prior to each water exchange and, if a radio-labelled compound is being used, the concentration determined by counting with a liquid scintillation counter (LSC). This value is then included in calculating the radioactivity budget (Section V, 2.2.1).
- (5) Potential losses of the test substance and transformation products to the atmosphere, either by volatilization or gaseous exchange, should be sampled weekly. Estimates of gas exchange rates with the atmosphere can be made by placing an inverted modified Pyrex glass dish (125 mm (diam) x 65 mm (depth)) 1-2 cm into the water (Figure 5) and drawing air from outside the test compartment (Figure 1) through the dish and then into a series of traps (see Figure 5). The first trap will capture organic volatiles and could be FlorisilTM, TenaxTM, etc. The second trap will collect CO₂, a final breakdown product of the test substance. The vigreux column (a component of the CO₂ trap) should contain an appropriate trapping material such as 0.5M NaOH. The air flow will be set at a designated rate and for a specified period of time. The column is then analyzed as per Section 4.4.4(8). The air flow rate through the modified glass dish from each microcosm is based upon the relative differences in air flow and surface area existing between the microlayer and test compartment (Section 3.1.1(6)). The air flow rate from the glass dish is calculated by:

Air flow rate of

**ERL-N STANDARD OPERATING PROCEDURE
MEASUREMENT OF THE ECOLOGICAL EFFECTS,
FATE, AND TRANSPORT OF CHEMICALS IN A
SITE-SPECIFIC EXPERIMENTAL MARINE MICROCOSM**

ERL-N SOP 1.03.020
REVISION 0
DECEMBER 1990
PAGE 13 OF 21

$$f = \frac{\text{test compartment}}{\text{Surface area of test compartment}} \times \begin{array}{l} \text{Surface area of inverted glass dish} \\ \text{plus surface area of air-water interface} \\ \text{contained by the glass dish} \end{array}$$

Gas transport from the dish is measured continuously between water turnover simulations.

- (6) The amount of test substance weakly adsorbed to the glass surfaces is measured during the cleaning process (see section 4.4.1(4)). This measurement is performed at each cleaning. At the end of the exposure period the glass surfaces are rinsed with an appropriate solvent to determine more strongly-bound quantities of the test substance (Section 4.4.6).
- (7) All samples for chemical analysis should be processed immediately or be handled and stored appropriately to prevent losses by microbiological degradation, chemical reaction, volatilization, or adsorption.
- (8) Standardized analytical methods should be used in performing the analysis to develop chemical and physical data. Appropriate sources for such methodology include, but are not limited to AOAC, 1975; APHA *et al.*, 1975; ASTM, 1979; USDI, 1977; USEPA 1974 and 1979. In general, atomic absorption analysis for metals and gas chromatography for analysis of organic compounds are preferable to colorimetric methods. However, in keeping with the recommendation that radiolabelled compounds be utilized for tracing the distribution of the test substances in the experimental microcosms, it is also recommended that liquid scintillation counting (LSC) be utilized for quantification and that high pressure liquid chromatography (HPLC) be used for the concentration, separation and, if possible, characterization of the test substance and transformation products of more highly water soluble compounds.

4.4.5 Ecological Effects Sampling

- (1) During the operation of the definitive test, the plexiglass covers of the test facility (Figure 1) are installed and all biological and chemical samples collected through the removable sampling ports.
- (2) All samples should be taken from approximately 10 cm below the water surface and away from the side of the tank. Sampling must be performed with the paddle system in operation.
- (3) Biological sampling should always precede the water exchange on days when the exchange is scheduled.
- (4) The following ecological information is considered to be essential:
 - (a) Phytoplankton abundance in numbers/ml per species.

**ERL-N STANDARD OPERATING PROCEDURE
MEASUREMENT OF THE ECOLOGICAL EFFECTS,
FATE, AND TRANSPORT OF CHEMICALS IN A
SITE-SPECIFIC EXPERIMENTAL MARINE MICROCOSM**

ERL-N SOP 1.03.020
REVISION 0
DECEMBER 1990
PAGE 14 OF 21

- (b) Zooplankton abundance in numbers/liter per life stage per species.
- (c) Identification of all benthic organisms reported in numbers/m² per species.
- (d) Ammonia levels in $\mu\text{g-at N/liter}$.

Other or additional analyses such as bacteria counts or growth measures of benthic organisms may be performed depending on conditions in the natural system being simulated and the analytical resources available to the investigators.

- (5) The type of biological sample, volume of sample, collection frequency, method of collection and analysis are listed below. Sampling should coincide with chemical fate sampling.
 - (a) Using an Eppendorf pipette, two-ml phytoplankton samples are collected daily during the mixing cycle and the number of organisms counted and identified according to the methods of Utermohl (1931).
 - (b) Zooplankton samples are collected twice weekly. A vacuum pump connected to a graduated separatory funnel which, in turn, is connected to the microcosm tank by a glass tube, allows a rapid non-destructive collection of two liters of water (Figure 6). The speed of sample collection must be great enough to overcome the avoidance reactions of the zooplankters. The two liter sample is passed through a 28 μm mesh nylon screen. The organisms retained on the screen are rinsed into a Petri dish and preserved. Numbers and species composition are determined by examination under a dissection microscope. Waste water from the two liter sample is pumped into a holding tank for subsequent analysis of the test substance.
 - (c) Water samples are analyzed for ammonia using a modification of the method of Bower and Holm-Hansen (1980). Samples are analyzed colorimetrically (at 640 nm) within two to three hours of collection. A standard curve is generated using standards of 2, 10, 20 and 30 $\mu\text{M NH}_4\text{Cl}$. Whereas the published method requires a 25ml sample and a 10 cm cuvette, for ammonia levels of 2 μM or greater it is recommended that a 10ml sample and a 1 cm cuvette be used. Reagent strengths are reduced proportionally. For ammonia concentrations below 2 μM , the method of Bower and Holm-Hansen (1980) is used without modification.
- (6) Estimates of sediment resuspension from the benthic subsystem are obtained indirectly by daily measurements of particulates that settle to the bottom of the microcosm. Previous studies have shown that up to 75% of these settleable particulates originated in the sediment. During water exchange, all settled particulates are removed by a sampling device similar to that used for zooplankton sampling (Figure 6). Five hundred-ml aliquots of this water-particulate homogenate are removed for filtration on a 0.4 μm filter, and then dried and weighed.

4.4.6 Procedure for Final Microcosm Sampling

- (1) At the time of final sampling (days 10, 20 and 30 for randomly chosen tanks; Section 4.4.1(3)), all tanks must have the tank and benthic box surface film (if present) removed and the water drained before taking the following samples.
 - (a) Triplicate sediment sub-cores (2.5 cm diam. x 7 cm deep) to determine the vertical distribution of the test substance in the benthic substrate. These cores include the sediment and associated biota.
 - (b) A representative sample of benthic organisms are taken from the benthic core so concentrations of the test substance and transformation products in those organisms can be determined.
 - (c) Identification and enumeration of all benthic organisms retained on a 0.5 mm screen is performed following the careful extrusion of the remaining sediment through the BOTTOM of the benthic cylinder. Density values are adjusted to compensate for the loss of organisms and sediment surface area due to sampling for chemistry (4.4.6(1a,b)).
 - (d) Measurement of the amount of test substance on the walls of the microcosm tanks. All glass surfaces except the inside of the benthic chamber are solvent rinsed two times to extract any remaining test substance. Following removal of the sediment from the benthic chamber, the empty glass cylinder is turned on its side and a small glass cylinder is sealed (using an O ring) against the inside of the large glass cylinder between the former sediment and water surface levels. This small chamber is then solvent rinsed twice and the solvent is analyzed for test substance. The amount of test substance removed from this small area is extrapolated to the amount adsorbed to the interior of the benthic chamber, and added to the value determined for the remainder of the microcosm.

4.5 Waste Disposal

- 4.5.1 Liquid wastes generated through sampling and water turnover must be collected and held until cleanup treatments have sufficiently reduced the chemicals and radioactivity to acceptable levels for disposal as per EPA (1989) and Nuclear Regulatory Commission (NRC, 1989), if using radio-labeled compounds.
- 4.5.2 Suggested techniques for waste water cleanup are:
 - (a) Filtration
 - (b) Activated charcoal
 - (c) Ion exchange
- 4.5.3 Contaminated solid wastes such as sediment, filters, charcoal, ion exchange resin, glassware, gloves, etc, must be packaged and disposed of in accordance with existing EPA and NRC regulations (EPA 1989, NRC, 1989).

IV. TROUBLE SHOOTING

Discussed in Methods Section.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Use of Test Results

- 1.1 The data generated from these microcosms will be used to better predict the potential fate, transport and ecological effects of the test substance in the marine system being simulated, and perhaps other similar systems as well. The assessment should discuss the potential ecological effects and possibility of indirect human exposures (i.e., via trophic transfers) associated with the mode and form of release of the test substance in a specific marine ecosystem.

2. Statistical Analysis

2.1 Ecological Effects Analysis

Multivariate, followed by univariate, analyses of variance (ANOVA; Steele and Torrie 1960) and regression techniques (Snedecor and Cochran 1980) should be used to analyze all data. The level of significance is set at the 5% level ($\alpha = 0.05$) unless otherwise defined. Documentation of all analysis used in the study must be provided.

2.1.1 Carrier Effects

Statistical comparisons between the carrier and sea water controls are necessary if a carrier, other than sea water, is used to add the test material to the experimental microcosms.

- (1) The method of repeated measures (ANOVA) should be used to establish whether the carrier has an effect on any of the ecological variables measured with time. Depending upon the repeated measures analysis, means and variances for the entire 30-day experiment should be determined and examined for differences using a pairwise comparison procedure such as the Studentized Range Test (Snedecor and Cochran p. 234, 1980). For those variables which are observed at one time interval (e.g., numerical abundance of benthic species) a pairwise comparison test should also be performed to detect differences between the carrier and sea water controls.

2.1.2 Microcosm Effects: Unperturbed Case

The type of comparisons used for establishing the relationship between the control microcosms and the natural system should be based on the differences between the carrier and sea water (carrier-free) controls.

- (1) If the responses from the carrier controls are statistically the same as that expressed in the sea water controls, then both control sets can be combined for purposes of quantifying the differences between laboratory controls and the natural system.
- (2) If the responses from the carrier controls differs statistically from that expressed in the sea water controls, only the sea water controls should be used to quantify the differences between laboratory controls and the natural system.

2.1.3 Test Material Effects: Perturbed Case

The model of repeated measures analysis of variance or a completely random design will be used to detect differences in the 30 day responses due to the test substance, relative to the laboratory control set. The specific replicates for the laboratory control set will depend on the results of Section V, 2.1.2.

2.2 Chemical Fate Analysis

The analysis should consist of two phases. First, calculation of a budget of all radioactivity for each concentration of the test substance with statistical analyses (ANOVA) to determine differences between the various compartments. Second, conversion of the radioactivity measures to weight of the test substance and breakdown products in the various compartments (Section V, 2.3) of the laboratory microcosms. The investigators should establish whether the means and associated variances of each variable are independent of one another. If dependence is found, then appropriate transformations (e.g., Log_{10}) should be applied so that the independence assumption can be satisfied. Statistical analyses (ANOVA) are performed on the transformed data to test for differences in exposure concentrations.

2.2.1 Radioactivity Budgets

The computation of a complete budget of all radioactivity is required. The budget must include: (a) the radioactivity added and/or removed either actively by investigator manipulation or gas transport, and (b) the distribution of radioactivity within the microcosms.

(1) Total Radioactivity Added

These additions consist of the amount of radioactivity added initially, in all subsequent water turnover additions and any additions due to cleaning losses.

(2) Total Radioactivity Removed

Removals of radioactivity consist of losses from water exchange (water and surface film), cleaning (i.e. sorbed to the surfaces of the glass holding vessel),

**ERL-N STANDARD OPERATING PROCEDURE
MEASUREMENT OF THE ECOLOGICAL EFFECTS,
FATE, AND TRANSPORT OF CHEMICALS IN A
SITE-SPECIFIC EXPERIMENTAL MARINE MICROCOSM**

ERL-N SOP 1.03.020
REVISION 0
DECEMBER 1990
PAGE 18 OF 21

settleable particulates and losses to the gas phase.

(3) Radioactive Budget in Microcosm at Time, t

The following relationship will be used to budget the radioactivity in a microcosm at time, t:

$$\text{Total activity in tank at time, } t = (\text{total activity added at time, } t) - (\text{total activity lost from tank at time, } t)$$

Equation 1

and, at the end of 30 day:

$$\text{Total activity in tank} = \text{total glass surfaces activity} + \text{total dissolved water column activity} + \text{total particulate water column activity} + \text{total surface microlayer activity} + \text{total benthic biota activity (large organisms not included in analysis of sediments, 4.4.6)}.$$

Equation 2

- (4) The sum of the measured activities from all compartments (equation 2) should be statistically compared (difference T-test; Steele & Torrie 1960) to the expected value derived from equation 1. If differences exist, then significant budgeting deficits have occurred at some point in the experiment. Previous studies utilizing these systems have accounted for >95% of the total quantity of test substance added.

2.3 Fate of Test Substance and Transformation Products

For each concentration, determine the steady state total weight of the test substance and transformation products in the following microcosm compartments:

- (a) Water column (particulate and dissolved fractions)
- (b) Surface film (if formed)
- (c) Sediment core
- (d) Glass surfaces

If the time to reach steady state is greater than 30 days for any of the compartments, regression techniques are used to approximate the steady state concentration or total mass of the test substance per microcosm (e.g., asymptotic regression model, p. 395, Snedecor and Cochran 1980).

2.3.1 Fate Analysis

Test for concentration differences in the mean steady state weight of the test substance and transformation products for each of the compartments listed in Section V, 2.3. In addition, test for compartmental differences in the mean steady state weights within each concentration.

2.4 Transport of Test Material and Transformation Products

Determine the steady state weight of the test material and transformation products exported from the gas, liquid and surface film compartments. In addition, compute the total weight of the test material and transformation products exported during the experiment. If the time to reach steady state is greater than 30 days for any of the compartments, use regression techniques to approximate the steady state levels for each microcosm.

2.4.1 Transport Analysis

Test for concentration differences in the mean steady state weight or concentration of the test substance and transformation products for each of the compartments listed in Section V, 2.4. In addition, test for compartment differences in the mean steady state weight or concentration within each treatment condition. Perform the same analysis as above for the integrated weight of the test substance and transformation products transported during the 30-day experiment.

2.5 Bioaccumulation

Choice of species should be based upon known or suspected linkages to man-based food chains. Compute both: (a) the concentration of the test substance and transformation products, in either the organisms and/or their specific tissues, in units of weight/kg wet weight and (b) the ratio of animal or tissue concentration to medium (sediment or water) concentration (Bioaccumulation Factor). Wet tissue weight, as opposed to dry weight, is recommended because of the low biomass of the types of species expected in microcosms of this scale. At least three replicates, (each from separate microcosm systems) of a given species/concentration combination should be measured. Indicate suspected feeding type in the case of benthic species.

2.5.1 The exposure medium for bioaccumulation calculations of a species will be dependent upon not only the water column or sediment concentrations of the test substance, but also on the levels found in food organisms. It is difficult to define the exposure history from these two types of exposure medium. As a result, estimates of bioaccumulation may be subject to question.

2.6 Ecological Risk Assessment of Test Substance

At present, there is no commonly accepted method for performing ecological risk analysis (Barnthouse *et al.* 1982; Barnthouse *et al.* 1986; Norton *et al.* 1988).

However, the quotient method is currently being used by EPA for premanufacture notice chemicals. This method utilizes exposure and biological effect information usually collected from isolated single species toxicity tests. Concentrations of the test substance at which biological effects occur are related to an expected exposure concentration. If the ratio of biological effects concentration to expected exposure concentration is one or less, it is assumed that no ecological impacts will occur. If, however, the ratio is much greater than one, impacts would be expected to occur. The use of the microcosms described herein can be viewed as performing an ecological risk assessment at least for the contained communities. If ecological effects are observed in the microcosm for any of the realistic exposure concentrations, one could assume similar types of effects or impacts would be observed in the natural system. The validity of such impact predictions are based upon (1) the biological correspondence between the controls and the natural field system being simulated (see Section V, 2.1.2), and (2) the accuracy of the mode and form of entry of the test substance into marine systems (see Section 4.3.1).

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**ERL-N STANDARD OPERATING PROCEDURE
MEASUREMENT OF THE ECOLOGICAL EFFECTS,
FATE, AND TRANSPORT OF CHEMICALS IN A
SITE-SPECIFIC EXPERIMENTAL MARINE MICROCOSM**

**ERL-N SOP 1.03.020
REVISION 0
DECEMBER 1990
PAGE 21 OF 21**

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I. OBJECTIVE

II. NECESSARY MATERIALS AND EQUIPMENT

A. Laboratory Processing of Field Collected *Ampelisca*

- large (32" diameter) 0.5 mm sieve
- large (32" diameter) 2.0 mm sieve
- one, 1.0 mm and one, 1.5 mm sieves
- 0.75 mm screen
- two aquarium fish nets
- four large Carolina (or similar) dishes
- small specimen cups, one for each holding jar
- squeeze bottle containing seawater
- two pipettes with bulbs
- holding jars.

B. Holding of *Ampelisca* Prior to Testing (Static-Renewal)

- As above.

C. Press Sieving Sediment

- 12", 2.0 mm sieve
- 12", 1.0 mm sieve
- round plastic bin
- Plexiglas paddle
- nylon spoon and spatula
- seawater for rinsing sieves, bin, etc.
- safety gloves, lab coat, safety glasses

D. Preparing Test Chambers

- seawater (not more than 48 hrs old)
- test chambers and lids
- labeling tape
- waterproof marker
- randomization sheet

E. Adding Sediments to Test Chambers

- sediment sample(s)
- control sediment

- turbulence reducer(s)
- plastic spoons and spatulas
- sponge
- squeeze bottle filled with seawater
- randomization sheet
- modified funnels
- electric drill and Teflon coated paddle

F. Sieving *Ampelisca* from Holding Jars

- two, 12" 0.5 mm sieves
- two, aquarium fish nets
- two, large Carolina dishes
- same number of transfer containers (plastic dishes) as holding jars
- same number of specimen cups as holding jars
- large plastic tray with drains
- squeeze bottles filled with seawater

G. Counting *Ampelisca* into Test Chambers

- *Ampelisca* from holding jars (see F)
- pipettes with bulbs
- one specimen cup for each test chamber and reference toxicant replicate
- clean seawater
- "specimen cup" sieve
- squeeze bottle with seawater

H. Daily Checking Amphipod Test

- lab coats, latex gloves, safety glasses

I. Sieving *Ampelisca* for Test Breakdown

- 0.5 mm sieve
- plastic grate
- plastic bin/basin
- bucket with drainage hole
- large Carolina dish
- seawater squeeze bottle
- pipette
- forceps

J. Picking Amphipods at End-of-Test

- lab coats, latex or vinyl gloves, and safety glasses

III. METHODS

A. LABORATORY PROCESSING OF FIELD COLLECTED *AMPELISCA*.

Field collected *Ampelisca abdita* are sieved out of their tubes and sediment in order to be sized and counted. The amphipods are then held in press sieved Narrow River sediment for at least 48 hrs before being used in toxicity test.

1. Assemble all material for sieving.
2. Sieving out field collected *Ampelisca*
 - a. Rinse and half-fill Carolina dishes with ambient temperature seawater.
 - b. Spray ambient temperature seawater into field collection bucket to loosen the amphipods from the sediment.
 - c. Pour half of a bucket's contents (tubes and sediment) onto large 2.0 mm sieve which is inserted into the large 0.5 mm sieve. (Sieves are set up over a settling bin to catch sediment.
 - d. Spray the material on the sieve with moderate force by pinching the end of the hose to loosen the amphipods from the tubes and sediment.
 - e. Remove the 2.0 mm sieve.
 - f. Vigorously shake and bounce the 0.5 mm sieve up and down (works best with 2 people).
 - g. Gently lower the 0.5 mm sieve into the water without completely submerging it so that amphipods float. Skim the amphipods from the surface of the water within the sieve using an aquarium net and transfer the amphipods to a Carolina dish. Repeat the shaking and skimming process until only a few amphipods remain in the sieve.
 - h. Pour more material into the sieve from the field bucket and repeat the above process; occasionally cleaning debris and tubes out from the 2.0 mm sieve.
3. Sizing *Ampelisca*
 - a. Rinse and half-fill three Carolina dishes. Label them S, M, and L.
 - b. Stack the sieves in ascending order (0.5 mm on bottom, 1.0mm in the middle and 1.5 mm on the top - a 0.75 mm screen may be placed between the 0.5 and 1.0 mm sieves.)
 - c. Pour collected amphipods slowly onto the 1.5 mm sieve.
 - d. Thoroughly, but gently, rinse the amphipods through the stacked sieves to separate them by size.
 - e. Using a squeeze bottle, rinse each sieve into a different Carolina dish. If a 0.75 mm screen is used rinse these amphipods into the Carolina dish labeled S instead of the animals from the 0.5 mm sieve.
4. Counting out *Ampelisca*.
 - a. Take out as many specimen cups as you have holding jars.
 - b. Rinse and half-fill specimen cups with seawater.
 - c. Randomly pipette 10 medium size amphipods (those from the 1.0 mm sieve) into each specimen cup until each cup contains a maximum of 350 amphipods. If there are not enough medium size amphipods, use the small (0.75 mm) amphipods can be added until 350 is reached.
 - d. Make a note of the number of animals/holding jar for later calculation of holding jar mortality.
5. Adding the amphipods to the holding jars (see section II.B for setting up holding jars).
 - a. Turn off the air leading to the holding jars and gently remove approximately 150 ml of water from each jar.

- b. Empty one specimen cup of amphipods into each holding jar; swirling cup to break up any clumps of amphipods. Push amphipods stuck on the air-water interface down with fingers.
- c. Wait a few minutes for the amphipods to swim down to the sediment surface then turn the air to the jars back on.

6. Clean all equipment with fresh water.

B. HOLDING OF *AMPELISCA* PRIOR TO TESTING (STATIC-RENEWAL).

Field collected *Ampelisca abdita* are held under static conditions. They should be held in gallon containers for at least 48 hrs (but no longer than 10 days) under test temperature and salinity conditions before being used for toxicity testing. Seawater is renewed (approximately 75%) and animals fed each weekday. No renewal or feeding is done on the weekends.

1. Holding jars should be set up 24 hours before animals are collected. They are placed in a controlled temperature culture box or waterbath, and aerated.
2. Place approximately 4 cm of Narrow River sediment into gallon jar and place jar into bucket. Rinse down sides with seawater from squeeze bottle or low pressure seawater line.
3. Place airline with attached pipet into holding jar so that it is approximately 6cm below the water surface. Use gentle aeration.
4. Seawater renewal and feeding (seawater renewal should take place in the morning and feeding in the afternoon)
 - a. Remove airlines.
 - b. Check for any emerged amphipods, if any gently prod down to encourage burrowing.
 - c. Siphon off approximately 75% of water volume.
 - d. Refill with clean seawater (for renewal) or 1 liter of diatom culture, *Phaeodactylum*, and clean seawater (for feeding), using a turbidity reducer so as not to disturb tubes or sediment.
 - e. Replace airlines.
5. Monitor temperature daily. Amphipods must be acclimated to 20°C.
6. Temperature can be increased no more than 3°C per day and animals should be maintained at 20°C for 48 hrs prior to use in test.

C. PRESS SIEVING SEDIMENT

Sediment samples are press sieved through a 2.0 mm sieve in order to remove large debris or predators. If a sample contains amphipods, the sample is press sieve first through a 2.0 mm and then a 1.0 mm sieve in an attempt to remove the resident amphipods.

1. Assemble materials for press sieving.
2. Wear appropriate safety gloves, lab coat and eye protection.

3. Pour, or scoop with nylon spoon, entire contents of sample container into sieve. DO NOT ADD ANY WATER!
4. Push sediment through 2.0 mm sieve with Plexiglass paddle or nylon spoon. NOTE: If sample contains resident amphipods stack the 2.0 mm sieve on top of the 1.0 mm sieve and press sediment through sieves.
5. Rinse out sample container with seawater into settling tank.
6. Homogenize sieved sample by stirring and return sample to original container or add to test chambers.
7. Label sample container "PRESS SIEVED", and date of sieving.
8. Between each sample, rinse all equipment with seawater into settling tank. After the last sample has been sieved rinse all equipment with tap water and then with DI water. Follow "glassware" SOP for final clean-up.

D. PREPARING TEST CHAMBERS.

Test chambers are quart-size canning jars with metric markings. A small glass dish with a hole drilled for an air line is used as a lid. Test chambers are prepared the day before sediments are added.

1. Assemble materials needed.
2. Fill out randomization sheet by picking numbers (1 through N, where N = the total number of jars for the test series) out of container. Sign and photocopy randomization sheet and place original in appropriate folder.
3. Label test chambers and lids with colored tape and waterproof marker. (Number 1 through N). On the lids; circle the numbers that correspond to the first two replicates of each group. These two replicates will be used for physical data measurements.
4. One other person must check the randomization sheet and sign.
5. Fill test chambers with seawater and cover. Allow to soak overnight.

E. ADDING SEDIMENTS TO TEST CHAMBERS.

1. Sediments are added to test chambers the day before the animals are added. Wear appropriate safety gloves, lab coat and eye protection.
2. Assemble all materials needed.
3. Homogenize previously press sieved sediment sample using an electric drill and a Teflon coated paddle. (Not all samples will have been previously press sieved, see section C for this procedure.)

4. For each sediment sample check the randomization sheet and select the appropriately numbered jars for that sample. Record the necessary information on the randomization sheet (ie. sample # description)
5. Pour or scoop ca. 200 ml of homogenized sediment through a modified funnel into each test chamber (quart size canning jars with metric markings). Gently tap test chamber or smooth sediment surface with a spatula to eliminate air pockets.
6. Rinse all mud from sides of test chamber using a squeeze bottle filled with seawater.
7. Using a turbulence reducer, slowly add ca. 600 ml of seawater to test chamber and place cover on chamber.
8. Transfer test jars to waterbath table.
 - a. place test chambers in table in numerical order, in groups of five (to make air tube easier to connect).
 - b. Put pipettes in test chambers so that the tip of the pipette is approximately halfway down the water column (between the 400 and 600 ml mark).
 - c. Attach air lines and turn on air pump.
 - d. Check all test chambers to make sure air is bubbling through pipettes.
 - e. Adjust 'gang' valves for gentle aeration, if necessary.

F. SIEVING *AMPELISCA* FROM HOLDING JARS.

Although animals are kept at ETC, the holding jars are sieved at ERLN.

1. Assemble material needed for sieving.
2. Place large plastic tray onto the cinder blocks in trough.
3. Fill Carolina dishes half full with seawater.
4. Carefully pour off a small amount of water before bringing holding jar to trough.
5. Over the large plastic tray, pour contents of holding jar into sieve and sieve as usual.

NOTE: Try to spray all tubes individually, so that very few animals remain in tubes.

 - a. Repeat process until no amphipods come out.
 - b. Rinse down tubes and place into specimen cup. DO NOT CAP.
 - c. Pour pods from dish into net over sieve and then rinse into transfer container. DO NOT CAP.
 - e. Rinse net into sieve to ensure all animals have been retrieved.
6. Do this for each holding jar and be sure that the animals from each are kept in separate transfer containers (to keep track of any mortality).
7. Rinse all equipment with deionized water. This includes holding jars and buckets.
8. Return all equipment to appropriate storage area.

9. Cap all transfer containers and specimen cups for transport to ETC for use.
10. At ETC pour contents of jars into separate, labelled Carolina dishes and aerate.

G. COUNTING *AMPELISCA* INTO TEST CHAMBERS.

Under normal testing procedures, 20 animals are placed into each test chamber.

1. Assemble materials needed.
2. Count out the number of specimen cups needed; one for each test chamber, one for each reference toxicant replicate, one for later sizing of the animals, and a few extra for dead and gravid animals. Fill each with approximately half full of seawater.
3. Determine initial amphipod mortality of holding jars, for each jar used.
 - a. Remove all suspected dead amphipods, determining condition under a stereomicroscope.
 - b. Record only the number of dead, if greater than 5% mortality animals from that jar should not be used for testing.
4. Adding animals to specimen cups; 20 into each (some from each holding jar).
 - a. Determine the number of animals that can be used from each holding jar by dividing 20 by the number of holding jars.
 - b. Using a pipette select healthy looking, non-gravid amphipods two or three at a time, and place into specimen cups.
 - c. Switch Carolina dishes and add 2-3 more amphipods to each cup.
 - d. Continue switching dishes until each cup contains 10 amphipods. Separate the number of cups needed for the reference test.
 - e. Continue to switch dishes until all remaining cups contain 20 animals. (If there are not enough animals the test may be performed with as few as 15 animals if the client agrees; check with lab manager).
5. Adding animals to test chambers.
 - a. Gently pour contents of specimen cup into a screened bottom transfer cup.
 - b. Verify amphipod count and check again for gravid females and remove, add additional amphipods from Carolina dishes if needed.

H. DAILY CHECKING AMPHIPOD TEST

Daily observations are made on each test container to check for emerged or dead amphipods and the presence of any molts. Salinity, pH and dissolved oxygen are recorded twice during the test; once on day one and again near the end of the test (preferably on day nine).

1. Make sure lab coats, latex gloves, and safety glasses are worn while checking test.
2. Check temperature recorder and note any irregularities. Read regular thermometer and record temperature.
3. Check test jars for emerged amphipods and molts.

- a. Remove pipette and lid from test chamber.
- b. Rinse the inside edge of chamber with seawater from squeeze bottle.
- c. Look into chamber for emerged amphipods or molts and remove with a clean pipette.
- d. Place amphipods/molts into petri dish half and examine under dissecting microscope.
- e. Classification of emerged amphipods.
 - Dead*--usually exhibit the following: not curled up; body is soft; gut is empty; may be disintegrating; when gently touched with a probe, the legs and antennae do not move; and there is no neuromuscular twitch.
 - Neuromuscular twitch (NMT)*--appears dead, but when gently touched with probe near the legs or midsection, one or two legs may kick spasmodically.
 - Emerged*--any live amphipod not burrowed in the sediment, i.e. floating, swimming, or lying on the surface of sediment.
 - Molt*--usually exhibits the following: transparent; no eyes; no gut; and appears hollow.
- f. Return emerged and NMT animals to test chambers; dispose of molts and dead animals. Record data on daily data sheets.
- g. Replace lid and pipette on chamber.
- h. Between each test chamber, rinse sampling pipette inside and out with seawater. When all chambers have been checked, rinse probes, pipettes, etc. with deionized water and let air dry on a paper towel. Wipe down microscope and turn off light.

4. On days one and nine check dissolved oxygen concentration, pH and salinity in all pre-selected jars.

5. End test on day 10. Check test as usual except emerged and nmt animals are placed into corresponding vials (instead of being returned to jars) and indicated on breakdown sheet. Rinse tools used to check test and placed in dirty dish bin.

I. SIEVING *AMPELISCA* FOR TEST BREAKDOWN

When ending the 10 day sediment toxicity test, the individual test chambers must be sieved out and picked through in order to determine survival.

1. Wear appropriate safety gloves, lab coat and eye protect.
2. Assemble all materials in sieving area (one/ sieving station)
3. Select a test chamber and corresponding medium Carolina dish. Sieve out reps 1-3 first.
4. Empty test chamber into sieve over the bucket.
5. Rinse the sediment through sieve using a moderate force tap water spray, then rinse the remaining material in sieve with seawater squeeze bottle.

6. Place the labeled medium Carolina dish inside the large Carolina dish. Use the seawater squeeze bottle to rinse the material from the sieve into the medium Carolina dish. Check the large Carolina dish for any spillage, and pipette or pour into medium Carolina dish.

7. Gently submerge the sieve to make sure no pods or tubes remain on the sieve. Use a pipette to transfer amphipods to the Carolina dish, use forceps for the tubes. Repeat until all amphipods have been removed.

J. PICKING AMPHIPODS AT END-OF-TEST

1. Put on lab coat, latex or vinyl gloves, and safety glasses.
2. Select a sieved sample of sediment in medium sized Carolina dish.
3. Look for amphipods.
 - a. Look into Carolina dish for any floating amphipods.
 - b. Agitate sediment and water to get any loose amphipods up to surface.
 - c. Pour out excess water into another Carolina dish, making sure no amphipods escape.
 - d. Look for tubes.
 - e. Pick through sediment and tubes using a stereomicroscope, a small portion at a time, removing amphipods, putting them into a separate petri dish. Put this sediment into the Carolina dish use in section J.3.c.
 - f. Continue picking through sediment until all of sediment is gone.
 - g. Place all live amphipods into the appropriate scintillation vial with the minimum amount of seawater.
 - h. Carefully record the following data on Breakdown sheet before continuing to another sample.
 - i. Picker's initial and time completed picking.
 - ii. Jar number-corresponds to the dish number and vial number.
 - iii. Number of dead amphipods during test-cumulative dead on day 10.
 - iv. Number recovered-total number of amphipods found (# live + # dead).
 - v. Number of amphipods unaccounted for-number of amphipods not found.
 - vi. Total number of amphipods dead-# amphipods dead during test + # dead found during picking + # unaccounted for.
 - i. Return sediment to original labeled Carolina dish.
 - j. any sample with more than ten percent unaccounted for should be placed in the QA, for re-pick pile.
4. Quality Control/Quality Assurance re-pick of replicates with more than 10% of amphipods unaccounted for.
 - a. Choose sample that you did not pick originally.
 - b. Look for amphipods as above.
 - c. Place live animals in scintillation vial.
 - d. Write down data about repick of sample on breakdown data sheet.

- i. Number repicked-total number of amphipods found during repicking.
- ii. Count of vial-this will be done once all samples have been picked and QA'd.
- iii. Number unaccounted for-number of amphipods not found, this is done after the vial is counted.
- iv. Final number dead-# amphipods dead during test + # found dead during picking and repicking, + # unaccounted for. this is done after the vial is counted.

5. Once all samples are picked and QA'd, add either tap water or alcohol to the vials containing pods. Use tap if the vials can be counted within one hour, if not use alcohol. Record the count of vial, # unaccounted for, and final # dead.

6. Clean up picking space.
- a. Make sure light of microscope is turned off.
 - b. Put picking tools and dishes into dirty dish bin.
 - c. Wipe down spilled water and sediment.
 - d. Clean off microscope and light.
 - e. Put away safety clothing into appropriate places.

IV. TROUBLE SHOOTING

Included in METHODS section.

V. STATISTICAL ANALYSIS AND DATA USAGE

Describe the statistical tests performed on the data and how the data will be used (If this section does not apply to your method, then state this here).

VI. REFERENCES

No reference cited.

**ERL-N STANDARD OPERATING PROCEDURE
CLEANING OF EQUIPMENT FOR TRACE METAL
ANALYSIS**

ERL-N SOP 2.01.001
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PAGE 1 OF 2

POINT OF CONTACT:

Chemistry Group
US Environmental Protection Agency and
Science Applications International Corporation
c/o US Environmental Protection Agency

I. OBJECTIVE

All bottles, vials, pipette tips etc. that are used for metals analyses need to be cleaned in deionized water and acid before use. This SOP describes cleaning procedures for items frequently used in the laboratory.

II. NECESSARY MATERIALS AND EQUIPMENT

- Heated water bath (50 - 60 °C)
- Nitric acid--concentrated or 2N
- Source of deionized water

III. METHOD

The concentration of acid and the length of time required for cleaning will vary with the type of plasticware and its intended use. A good rule of thumb is to use an acid concentration stronger than the item will be exposed to or as strong as is possible without deterioration of the item. In all cases, cleaning is enhanced by heating at 50-60 C.

A. Concentrated nitric acid procedure

This cleaning procedure is recommended for pipette tips, 1 ml vials used for AA samples, and 7 ml vials used for storage of samples in 2N HNO₃. This procedure should be performed in a thick-walled hard plastic container. Caps and vials should be processed in separate containers.

1. Soak plasticware in concentrated HNO₃ for 24 hours in a heated water bath at 50-60°C.
2. Pour off acid and fill container with DI water. Soak again for 24 hours.
3. Repeat steps 1 and 2, then rinse with DI five times.

B. 2N Nitric Acid Procedure

This procedure is recommended for all soft plastic polyethylene bottles, and items made of polysulfone. In most cases the plasticware can be soaked in a larger plastic jug. However,

**ERL-N STANDARD OPERATING PROCEDURE
CLEANING OF EQUIPMENT FOR TRACE METAL
ANALYSIS**

ERL-N SOP 2.01.001
REVISION 0
JANUARY 1991
PAGE 2 OF 2

for bottles 250 ml or larger, the bottles can be filled to over flowing with the acid or water, being sure that the liquid is in contact with the cap.

1. Items are soaked for 48 hours in 2N HNO₃ at 50-60°C.
2. Items are then soaked in DI water twice for 24 hours each time.
3. Items are rinsed five times in DI water.
4. All items are then dried on a clean bench.

C. Nucleopore filters

Nucleopore membrane filters used for seawater filtrations are highly contaminated with metals, especially chromium. Therefore, they must be cleaned before use.

1. Soak the filters in 2N HNO₃ for five days at 50-60°C followed by five days in DI water.
2. Filters are then rinsed several times with DI water and stored in DI until used.
3. If the filters are to be weighed, they can be individually dried in plastic petri dishes that have been cleaned by the 2N HNO₃ procedure.

IV. TROUBLE SHOOTING

A. Concentrated nitric acid procedure

1. All surfaces that will be exposed to sample or reagent must be thoroughly cleaned; therefore, care must be taken to shake the containers several times to eliminate any air pockets that might be trapped inside vials.
2. The soak in concentrated acid should not last longer than 24 hours or the plasticware will begin to deteriorate.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
FIELD USE OF THE HIGH VOLUME SEAWATER SAMPLING
APPARATUS FOR ORGANICS ANALYSIS**

ERL-N SOP 2.01.002
REVISION 0
JANUARY 1991
PAGE 1 OF 4

POINT OF CONTACT:

Chemistry Group
Science Applications International Corporation
c/o US Environmental Protection Agency
27 Tarzwell Dr.
Narragansett, RI 02882

I. OBJECTIVE

The objective of this document is to describe the recommended field use of the high volume organic sampling apparatus, the materials required and relate information that may be useful in trouble shooting problems encountered.

II. NECESSARY MATERIALS AND EQUIPMENT

- High volume pump*
- Stainless steel coated hoses*
- Filter housing
- Foam plug housings (loaded with extracted plugs)
- Generator*
- Pre-combusted Type A/E glass fiber filters 293 mm
- Acetone rinsed stainless steel cans with tops
- TWO 18 L containers with DI water
- Labeling tape
- Lab marker
- Lab notebook
- Gloves (field gloves and plastic lab gloves)
- Large ziplock bags
- Teflon tape
- Duct tape
- Cooler with ice
- Forceps
- Spatulas
- Filter housing wrench
- Crescent wrenches 1 1/4" (2), 11/16", 1", 7/8"
- Two large adjustable wrenches
- One hammer

* This equipment is located in the field gear locker
on the hill

III. METHOD

**ERL-N STANDARD OPERATING PROCEDURE
FIELD USE OF THE HIGH VOLUME SEAWATER SAMPLING
APPARATUS FOR ORGANICS ANALYSIS**

ERL-N SOP 2.01.002
REVISION 0
JANUARY 1991
PAGE 2 OF 4

A. Preparation

1. If the pump, hoses, filter housing, and foam plug housings have not been recently used, they should be cleaned well with Alconox and tap water. It is possible, though extremely difficult, to set up the pump in the lab and circulate tap water through it. Any parts of the apparatus that can be, should be thoroughly rinsed with DI water prior to use.

Note: The stainless steel covering the hoses is frayed in some places. It is advisable to wear work gloves whenever manipulating them to avoid cutting your hands!!

2. Filters should be individually wrapped in clean aluminum foil and combusted in a 450°C oven for 6 hours. After the filters have been combusted it is extremely important that they not be bent, twisted or disturbed in any way. They should be taken out of the oven and immediately placed in a covered container in which they can remain until it is time for them to be used. There should one filter for each sample, one for each of two field blanks and at least three extra.

3. Filter containers (stainless steel cans with tops) should be washed, rinsed with DI water and cleaned with acetone.

4. The procedure for the preparation for the foam plugs is included in the s.o.p. for the analysis of dissolved organics using foam plugs. The housings should be wrapped in clean aluminum foil for transport to the field.

B. Field Use

1. The pump will float when placed in the water however, a safety line should be tied from it to the boat.

2. Pass the intake hose through the water filling it completely with water. This is necessary to prime the pump. Attach the intake hose to the pump.

3. Attach the outflow hose to the pump.

4. Start the generator and start the pump. There should be a strong flow of water out of the outflow hose.

5. Once the pump is primed, it may be turned off as long as the operators are careful not to allow air to enter the device. At this time, open the filter housing and very carefully place one filter on the screened platform. Hand tighten the screws and then completely tighten them with the filter housing wrench.

6. Attach the hose from the bottom of the filter housing to the top of the foam plug housing.

7. To take the field blank, place the end of the intake hose in the DI water container making sure not to introduce any air into the system. Start the pump for 5 seconds.

**ERL-N STANDARD OPERATING PROCEDURE
FIELD USE OF THE HIGH VOLUME SEAWATER SAMPLING
APPARATUS FOR ORGANICS ANALYSIS**

**ERL-N SOP 2.01.002
REVISION 0
JANUARY 1991
PAGE 3 OF 4**

Stop the pump. Attach the hose from the outflow of the pump to the top of the filter housing. Open the air bleed valve on the top of the filter housing. Start the pump. Shut the air bleed valve once the air stops coming out (approximately 5 seconds). There should be a trickle of water coming out of the foam plug.

8. Pump as much of the 18 liters of DI water as you can through the apparatus without getting any air in the system. This should take approximately 10-15 minutes. Turn off the pump. If the apparatus has not been used recently or was last used in a contaminated area it would be advisable to take another field blank before sampling the seawater. Place the intake hose in the second 18 liters of DI water before changing the foam plug and the filter. If not taking a second field blank, the intake hose may be place back into the seawater.

9. Open the air bleed valve on the filter housing. Unscrew the housing top and carefully remove the top. Examine the filter to see if it is intact. If it is, use the spatulas to fold the filter and place it in the stainless steel can. Label the can.

10. Replace the ends of the foam plug housing. Label the housing and wrap it in aluminum foil. Place the filter and foam plug on ice in the cooler.

11. To take a seawater sample, repeat steps 7-10 with the intake hose in the water. It is not necessary to stop the pump and attach the outflow hose to the filter housing as this is done already. A second hose may be attached to the outflow of the foam plug housing and the end placed in the empty 18L DI water container. This will make it possible to measure the volume of water sampled.

IV. TROUBLE SHOOTING

1. Pump is on, no water flow - The pump has not been primed properly. Purge the intake hose of air and reattach. Hold the outflow hose and the foam plug lower in the boat.

2. The filter housing leaks - Wipe standing water off of the top of the housing. Use the filter wrench to tighten the screws.

3. Leaks occur at hosing attachments - Use teflon tape to wrap the male connectors prior to use.

4. Filters break - Experience has shown the breaking filters usually are the result of rough handling. Place the next filter on and make sure to shield the housing and filter from the wind while putting the filter on.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

**ERL-N STANDARD OPERATING PROCEDURE
FIELD USE OF THE HIGH VOLUME SEAWATER SAMPLING
APPARATUS FOR ORGANICS ANALYSIS**

ERL-N SOP 2.01.002
REVISION 0
JANUARY 1991
PAGE 4 OF 4

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
SUBTIDAL SEDIMENT CHEMISTRY SAMPLING**

ERL-N SOP 2.02.002
REVISION 0
DECEMBER 1990
PAGE 1 OF 3

POINT OF CONTACT:

Timothy R. Gleason
Science Applications International Corporation
c/o Environmental Protection Agency
27 Tarzwell Drive
Narragansett, RI 02882

I. OBJECTIVE

Collection of subtidal sediments (cores and bulk) from research vessels for chemical and toxicological analysis.

II. NECESSARY MATERIALS AND EQUIPMENT

The materials and equipment required or available for sediment sampling may vary depending on the vessel used for sampling. The investigator should determine the deployment capabilities of the vessel to be used and the equipment available prior to planning the sampling event. The Smith-MacIntyre may be deployed from a vessel with suitable winches, and an A-Frame or Davit arrangement. The ponar grab is small enough to be deployed and retrieved by hand and is therefore suitable for use in small boats which lack hydraulic winches (though a winch is helpful).

Additional equipment needed:

- Smith-MacIntyre (0.1 m) or Ponar grab sampler
- Stand - for the grab
- Lead weights - 4 triangular lead weights for Smith-Mac (no weights for the ponar)
- Cocking bar for Smith-Mac
- Hard hats
- Face shield or other eye protection
- Respirator - if conditions dictate
- Standard safety equipment (ie-first aid kit)
- 1/2 - 3/4 line for the Ponar grab if a wire winch is not available
- Tub - into which to dump sediment
- Teflon scoop
- Buckets (2-3) - for rinsing
- Cable cutter
- Cable crimping tools
- Electrical tape
- Duct tape
- Tools - screw drivers, wrenches, hammer, other
- Sample containers - gallon jars, cleaned and acid stripped
- Coolers with ice, or other means of cold storage
- Water-proof sample notebook

**ERL-N STANDARD OPERATING PROCEDURE
SUBTIDAL SEDIMENT CHEMISTRY SAMPLING**

ERL-N SOP 2.02.002
REVISION 0
DECEMBER 1990
PAGE 2 OF 3

- Water-proof markers
- Suitable protective clothing (from weather as well as contaminants)
- Foul weather gear
- Boots
- Exposure suits (orange worksuits) for winter sampling
- Gloves - appropriate gloves for suspected conditions
- Compass - handheld for taking sitings at sample sites
- Line and twine
- Twine/pocket knife
- 6" polyethylene cores
- Core caps
- Gravity coring device
- 30" polyethylene cores
- Gravity core tripping mechanism

Whenever possible, back-up equipment should be carried on board.

III. METHODS

1. Skim the top 1-2 cm of the undisturbed grab sample using a clean teflon scoop for chemistry and toxicity analysis. Approximately five Smith-Mac grabs are needed to collect a one gallon composite sample. Rinse the grab and scoop with seawater between grabs. The grab sampler should be "washed-down" with the deck hose (or other means, ie. buckets) between stations.

2. Cores are typically taken one of two ways:

a. Taking a core from an undisturbed grab sample

1. Insert "small" (6") cores into the middle of an undisturbed grab sample.
2. Place a cap on the top of the core and remove the core from the grab.
3. Place the second cap on the core after removal from the grab.

b. Gravity cores (collected infrequently)

Gravity cores are used to take deeper (> 6") inch cores. The parts needed to assemble the gravity corer and it's tripping mechanism are contained in a large metal ammunition trunk which is generally stored at the Davisville warehouse. Because the gravity core is seldom used, it is strongly recommended that the corer be assembled at the laboratory to ascertain that all necessary parts are present and functional.

IV. TROUBLE SHOOTING

**ERL-N STANDARD OPERATING PROCEDURE
SUBTIDAL SEDIMENT CHEMISTRY SAMPLING**

ERL-N SOP 2.02.002
REVISION 0
DECEMBER 1990
PAGE 3 OF 3

When using the Smith-MacIntyre grab in very soft sediments it may necessary to attach plywood "shoes" to the landing pads. This is necessary to increase the sensitivity of the "firing" mechanism.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF FILTER SAMPLES FOR PCBS**

ERL-N SOP 2.03.001
REVISION 0
JANUARY 1991
PAGE 1 OF 3

POINT OF CONTACT:

Chemistry Group
US Environmental Protection Agency and
Science Applications International Corporation
27 Tarzwell Dr.
Narragansett, RI 02882

I. OBJECTIVE

The objective of this document is to define the standard operating procedure for analyzing filter samples for polychlorinated biphenyls.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware*

- 1 liter separatory funnels
- 100 ml centrifuge tubes
- 250 ml Erlenmeyer flasks
- 250 ml round bottom flasks
- Kuderna-Danish flasks
- snyder columns
- graduated concentrator tubes
- microsnyder columns
- ebulators
- screw-top vials
- 1 ml syringe
- pasteur pipets
- boiling beads

* all glassware is combusted at 450 C for 6 hours

B. Chemicals

- 1,1,2 Trichlorotrifluoroethane (Freon) - high purity
- Acetone - high purity grade
- Hexane UV - pesticide grade
- Heptane - high purity grade
- Methanol - high purity grade
- Methylene chloride - high purity grade
- Hydrochloric acid - 8 molar
- Sulfuric acid - concentrated
- Sodium sulfate - anhydrous
- Copper powder - spherical -325 mesh

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF FILTER SAMPLES FOR PCBS**

ERL-N SOP 2.03.001
REVISION 0
JANUARY 1991
PAGE 2 OF 3

C. General

- heating mantle for round bottom flasks
- heating mantle for concentrator tubes
- Kinematic polytron
- RC2-B refrigerated centrifuge

III. METHODS

1. Place the filter(s) into an acetone rinsed 100 ml centrifuge tube.
2. Add recovery standard (octachloronaphthalene) at a level equivalent to expected PCB concentrations in the sample.
3. Add 25 mls of acetone and polytron for 20 seconds at a setting of about five. Centrifuge for 10 minutes at 2500 rpm. Pour off extract into a 1 liter separatory funnel containing approximately 600 mls of freon rinsed deionized water. Repeat this once more with acetone and then one final time with 25 mls of freon.
4. Add 25 mls of freon to the separatory funnel and shake. Allow the layers to separate and draw off the freon from the bottom of the funnel into a 250 ml Erlenmeyer flask. Repeat this procedure twice with 50 ml of freon combining all the extracts in the Erlenmeyer flask.
5. Add anhydrous sodium sulfate to the combined extracts to remove any water.
6. Pour the sample into a round bottom flask containing 5-10 boiling beads, making sure to rinse the sodium sulfate three times with freon. The round bottom is then fitted with a K-D flask and snyder column and placed on a heating mantle. The solvent is volume reduced to about 10 mls and exchanged to hexane.
7. The sample in the round bottom is treated with activated copper (copper treated with 8M hydrochloric acid and rinsed with in this order DI water, methanol, methylene chloride and hexane) to remove any sulfur present.
8. Using a pasteur pipet, transfer the sample to a 10 ml graduated concentrator tube fitted with a microsnyder column and an ebulator. Be careful not to carry over any copper. Add 0.5 mls of heptane and reduce the volume to 0.5 mls. Add heptane to bring the final volume to 1 ml.
9. The 1 ml sample is transferred, using a pasteur pipet, to a screw-top vial to which is added approximately 0.5 ml concentrated sulfuric acid. The vial is rolled by hand for at least 30 seconds and the acid and sample are allowed to interact for a least 15 minutes.
10. The heptane layer is drawn off from the acid with a 1 ml syringe and stored in a

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF FILTER SAMPLES FOR PCBS**

ERL-N SOP 2.03.001
REVISION 0
JANUARY 1991
PAGE 3 OF 3

second 1 ml screw-top vial.

IV. TROUBLE SHOOTING

1. The polytroning is made easier by allowing the filters to remain in the centrifuge tube overnight with the acetone.
2. Emulsions can sometimes be broken up by adding a small amount of freon rinsed sodium chloride, shaking the separatory funnel and swirling gently.
3. The copper should turn black upon addition to the sample. Add sufficient copper until it retains its red color.
4. The sample should not be removed from the acid until it is completely clear.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF SEDIMENT SAMPLES FOR PCBS**

ERL-N SOP 2.03.002
REVISION 0
JANUARY 1991
PAGE 1 OF 3

POINT OF CONTACT:

Chemistry Group
US Environmental Protection Agency and
Science Applications International Corporation
c/o US Environmental Protection Agency
27 Tarzwell Dr.
Narragansett, RI 02882

I. OBJECTIVE

The objective of this document is to define the standard operating procedure for analyzing heavily contaminated (Superfund) sediment samples for polychlorinated biphenyls.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware*

- 1 liter separatory funnels
- 100 ml centrifuge tubes
- 250 ml Erlenmeyer flasks
- 250 ml round bottom flasks
- K-D flasks
- snyder columns
- graduated concentrator tubes
- microsnyder columns
- ebulators
- screw-top vials
- 1 ml syringe
- pasteur pipets
- boiling beads

* all glassware is combusted at 450 C for 6 hours

B. Chemicals

- 1,1,2 Trichlorotrifluoroethane (Freon) - high purity
- Acetone - high purity grade
- Hexane UV - pesticide grade
- Heptane - high purity grade
- Methanol - high purity grade
- Methylene chloride - high purity grade
- Sulfuric acid - concentrated
- Hydrochloric acid - 8 molar

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF SEDIMENT SAMPLES FOR PCBS**

ERL-N SOP 2.03.002
REVISION 0
JANUARY 1991
PAGE 2 OF 3

- Sodium sulfate - anhydrous
- Copper powder - spherical -325 mesh

C. General

- heating mantle for round bottom flasks
- heating mantle for concentrator tubes
- Model W-370 ultrasonic processor
- RC2-B refrigerated centrifuge
- Stabil-therm gravity oven
- aluminum weighing pans

III. METHODS

1. Place a 2 g aliquot of sediment into a pre-weighed aluminum pan for a wet/dry determination and dry in 120 C oven overnight or until the sediment has turned a uniform light gray color.
2. Place 0.2 to 1.0 g wet sediment, depending upon the expected level of contamination, into an acetone rinsed 100 ml centrifuge tube.
3. Add recovery standard (octachloronaphthalene) at a level equivalent to expected PCB concentration in the sample.
4. Add 25 mls of acetone and sonicate, at a level of 5, for 60 seconds. Centrifuge for 10 minutes at 2500 rpm. Pour off extract into a 1 liter separatory funnel containing approximately 600 mls of freon rinsed deionized water. Repeat this once with acetone and then one final time with 25 mls of freon.
5. Add 25 mls of freon to the separatory funnel and shake. Allow the layers to separate and draw off the freon from the bottom of the funnel into a 250 ml Erlenmeyer flask. Repeat this procedure twice with 50 ml of freon combining all the extracts in the Erlenmeyer flask.
6. Add anhydrous sodium sulfate to the combined extracts to remove any water.
7. Pour the sample into a round bottom flask, making sure to rinse the sodium sulfate three times with freon. The round bottom is then fitted with a K-D flask and snyder column and placed on a heating mantle. The solvent is volume reduced to about 10 mls and exchanged to hexane.
8. The sample in the round bottom is treated with activated copper (copper treated with 8M hydrochloric acid and rinsed with, in this order, DI water, methanol, methylene chloride and hexane) in order to remove any elemental sulfur present.
9. Transfer the sample with a pasteur pipet to a 10 ml graduated concentrator tube fitted with a microsnyder column and an ebulator. Be careful not to carry over any

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF SEDIMENT SAMPLES FOR PCBS**

**ERL-N SOP 2.03.002
REVISION 0
JANUARY 1991
PAGE 3 OF 3**

copper. Add 0.5 mls of heptane and reduce the volume to 0.5 mls. Add heptane to bring the final volume to 1 ml.

10. The 1 ml sample is transferred to a screw-top vial to which is added approximately 0.5 ml concentrated sulfuric acid. The vial is rolled by hand for at least 30 seconds and the acid and sample are allowed to interact undisturbed for a least 15 minutes.

11. The heptane layer is drawn off from the acid with a 1 ml syringe and stored in a second 1 ml screw-top vial.

IV. TROUBLE SHOOTING

1. Emulsions can sometimes be broken up by adding a small amount of freon rinsed sodium chloride, shaking the separatory funnel and swirling gently.

2. The copper should turn black upon addition to the sample. Sufficient quantities should be added until it retains its red color.

3. The sample should not be removed from the acid until it is completely clear.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF TISSUE SAMPLES**

**ERL-N SOP 2.03.003
REVISION 0
JANUARY 1991
PAGE 1 OF 3**

POINT OF CONTACT:

**Chemistry Group
US Environmental Protection Agency and
Science Applications International Corporation
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27 Tarzwell Dr.
Narragansett, RI 02882**

I. OBJECTIVE

The objective of this document is to define the standard operating procedure for analyzing heavily contaminated (superfund) mussel samples for polychlorinated biphenyls.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware*

- 1 liter separatory funnels
- 100 ml centrifuge tubes
- 250 ml Erlenmeyer flasks
- 250 ml round bottom flasks
- K-D flasks
- Snyder columns
- graduated concentrator tubes
- microsnyder columns
- ebulators
- screw-top vials (large and small)
- pasteur pipets
- boiling beads

* all glassware is combusted at 450 C for 6 hours

B. Chemicals

- 1,1,2 Trichlorotrifluoroethane (Freon) - high purity
- Acetone - high purity grade
- Hexane UV - pesticide grade
- Heptane - high purity grade
- Sulfuric acid - concentrated
- Sodium sulfate - anhydrous
- Copper powder - spherical -325 mesh

C. General

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF TISSUE SAMPLES**

ERL-N SOP 2.03.003
REVISION 0
JANUARY 1991
PAGE 2 OF 3

- heating mantle for round bottom flasks
- heating mantle for concentrator tubes
- Kinematic polytron
- RC2-B refrigerated centrifuge
- Stabil-therm gravity oven
- aluminum weighing pans

III. METHODS

1. Homogenize entire tissue sample with polytron.
2. Place 2 to 5 grams homogenized tissue sample into an acetone rinsed 100 ml centrifuge tube.
3. Add recovery standard (octachloronaphthalene) at a level equivalent to expected PCB concentrations in the sample.
4. Weigh approximately 1 g of homogenized tissue into an aluminum weighing pan for a wet to dry determination. Place pan in a 120°C oven overnight.
5. Add 25 mls of acetone and polytron for 20 seconds at a setting of approximately 5. Centrifuge for 10 minutes at 2500 rpm. Pour off extract into a 1 liter separatory funnel containing approximately 600 mls of freon rinsed deionized water. Repeat this step twice and combine all the extracts in the separatory funnel.
6. Add 50 mls of freon to the separatory funnel and shake. Allow the layers to separate and draw off the freon from the bottom of the funnel into a 250 ml Erlenmeyer flask. Repeat this procedure twice with 50 ml of freon combining all the extracts in the Erlenmeyer flask.
7. Add anhydrous sodium sulfate to the combined extracts to remove any water.
8. Pour the sample into a round bottom flask containing 5-10 boiling beads, making sure to rinse the sodium sulfate three times with freon. The round bottom is then fitted with a K-D flask and snyder column and placed on a heating mantle. The solvent is volume reduced to about 10 mls and exchanged to hexane.
9. Transfer the sample with a pasteur pipet to a 10 ml graduated concentrator tube fitted with a microsnyder column and an ebulator. Reduce the volume to 10 mls and remove 1 ml and place in a pre-weighed aluminum pan for a total lipid determination.
10. The remaining 9 mls are transferred to large screw top vial to which ~ approximately 10 ml concentrated sulfuric acid is added. The vial is rolled by hand for at least one minute and then the acid and the sample are allowed to interact undisturbed for at least 30 minutes.

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF TISSUE SAMPLES**

ERL-N SOP 2.03.003
REVISION 0
JANUARY 1991
PAGE 3 OF 3

11. The hexane layer is removed with a pasteur pipet and transferred to a 10 ml graduated concentrator tube fitted with a microsnyder column and an ebulator.
12. The sample is volume reduced using a tube heater and solvent exchanged to 1 ml of heptane.
13. The sample is stored in a 1 ml screw-top vial.

IV. TROUBLE SHOOTING

1. Emulsions can sometimes be broken up by adding a small amount of freon rinsed sodium chloride, shaking the separatory funnel and swirling gently.
2. The sample should not be removed from the acid until it is completely clear. Experience has shown that with mussel samples it is best to plan to allow the interaction with the acid to take place overnight.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF WATER SAMPLES FOR PCBS**

**ERL-N SOP 2.03.004
REVISION 0
JANUARY 1991
PAGE 1 OF 3**

POINT OF CONTACT:

**Chemistry Group
US Environmental Protection Agency and
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Narragansett, RI 02882**

I. OBJECTIVE

The objective of this document is to define the standard operating procedure for analyzing heavily contaminated (Superfund) water samples for polychlorinated biphenyls.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware*

- 2 liter separatory funnels
- 1000 ml graduated cylinder
- 250 ml Erlenmeyer flasks
- 250 ml round bottom flasks
- Kuderna-Danish flasks
- snyder columns
- graduated concentrator tubes
- microsnyder columns
- ebulators
- screw-top vials
- 1 ml syringe
- pasteur pipets
- boiling beads

* all glassware is combusted at 450 C for 6 hours

B. Chemicals

- 1,1,2 Trichlorotrifluoroethane (Freon) - high purity
- Hexane UV - pesticide grade
- Heptane - high purity
- Sulfuric acid - concentrated
- Sodium sulfate - anhydrous

C. General

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF WATER SAMPLES FOR PCBS**

ERL-N SOP 2.03.004
REVISION 0
JANUARY 1991
PAGE 2 OF 3

- heating mantle for round bottom flasks
- heating mantle for concentrator tubes

III. METHODS

1. Pour the 1 liter water sample from an ICHEM sample bottle to a 2 liter separatory funnel. Rinse the inside of the ICHEM bottle three times with freon and add the rinses to the sample in the separatory funnel.
2. Add recovery standard (octachloronaphthalene) at a level equivalent to expected PCB concentrations in the sample.
3. Extract the sample three times using 50 mls of freon each time and the extracts are collected in a 250 ml Erlenmeyer flask. Measure the volume of water in the separatory funnel before disposing.
4. Add anhydrous sodium sulfate to the combined extracts to remove any water.
5. Pour the sample into a round bottom flask containing 5-10 boiling beads, making sure that the sodium sulfate is rinsed three times with freon. The round bottom is then fitted with a K-D flask and snyder column and placed on a heating mantle. The solvent is volume reduced to approximately 10 mls and exchanged to hexane.
6. Transfer the sample using a pasteur pipet to a 10 ml graduated concentrator tube fitted with a microsnyder column and an ebulator. Then add 0.5 mls of heptane and reduce the volume to 0.5 mls. Remove from the heating mantle and add heptane to bring the final volume to 1 ml.
7. Transfer the 1 ml sample with a pasteur pipet to a screw-top vial and add approximately 0.5 ml concentrated sulfuric acid. Roll the vial hand for at least 30 seconds and allow the acid and sample to interact for a least 15 minutes.
8. The heptane layer is now drawn off from the acid with a 1 ml syringe and stored in a second 1 ml screw-top vial.

IV. TROUBLE SHOOTING

1. Emulsions can sometimes be broken up by adding a small amount of freon rinsed sodium chloride, shaking the separatory funnel and swirling gently.
2. The sample should not be removed from the acid until it is completely clear.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF WATER SAMPLES FOR PCBS**

**ERL-N SOP 2.03.004
REVISION 0
JANUARY 1991
PAGE 3 OF 3**

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
COLUMN CHROMATOGRAPHY OF SEMIVOLATILE
ORGANIC ANALYTES**

ERL-N SOP 2.03.005
REVISION 0
JANUARY 1991
PAGE 1 OF 3

POINT OF CONTACT:

Chemistry Group
US Environmental Protection Agency and
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I. OBJECTIVES

The objective of this document is to define the standard operating procedure for the preparation of columns for the cleanup and chemical class separation of semi-volatile organic compounds from marine samples. The extract fractions will be analyzed by gas chromatography (GC) or gas chromatography/mass spectrometry (GC/MS).

II. NECESSARY MATERIALS AND EQUIPMENT

- 9.5-mm ID X 45-cm glass chromatography column with 200 ml reservoir
- Top-loading balance capable of weighing to 0.01 g
- Turbo-Vap (Zymark) apparatus, with heated water bath maintained at 25-35°C
- Glass Turbo-Vap flasks, 200 ml
- Nitrogen gas, compressed, 99.9% pure
- Tumbler, ball-mill
- Glass graduated cylinders, 100- and 500-ml
- Glass beakers, 50-ml
- Borosilicate glass vials with Teflon-lined screw caps, 2-ml
- Micropipets, solvent rinsed or muffled at 400°C
- Reagents:
 - Pentane, pesticide grade or equivalent
 - Methylene Chloride (CH_2Cl_2), pesticide grade or equivalent
 - Hexane, pesticide grade or equivalent
 - Heptane, pesticide grade or equivalent
 - Deionized water, pentane-extracted
 - BioSil A silicic acid, 100-200 mesh
- Glass wool, silanized

III. METHODS

A. Silica gel preparation

1. Approximately 150 grams of fully activated silica gel is accurately weighed and

ERL-N STANDARD OPERATING PROCEDURE
COLUMN CHROMATOGRAPHY OF SEMIVOLATILE
ORGANIC ANALYTES

ERL-N SOP 2.03.005
REVISION 0
JANUARY 1991
PAGE 2 OF 3

transferred to a glass jar.

2. The silica gel is deactivated by adding 7.5% (weight basis) of pentane-extracted deionized water. The water is weighed accurately and an appropriate amount is added dropwise, ~ 1 ml at a time, to the silica gel. After each water addition, the jar is hand-shaken vigorously.
3. The glass jar is then placed on a ball-mill tumbler and allowed to tumble overnight.
4. After tumbling, the jar is removed from the tumbler. The silica gel is stored tightly sealed in the jar at room temperature until use.

B. Column preparation

1. The glass columns are set up in ring stands in a fume hood.
2. Glass wool, sufficient to create a 1 cm thick plug in the column is placed into the reservoir of the column. A glass rod is used to push the glass wool to the bottom of the column.
3. 11.5 g of the 7.5% deactivated silica gel is weighed out in a beaker. Approximately 30 ml of CH_2Cl_2 is added to the beaker to form a slurry. The slurry is then carefully poured into the column. The beaker is rinsed with additional CH_2Cl_2 , as are the inner walls of the reservoir to ensure all silica is introduced to the column. The total volume of CH_2Cl_2 should be approximately 50 ml.
4. The column is allowed to drip, with the eluate being collected and discarded. When the level of the CH_2Cl_2 just reaches the top of the silica gel, 50 ml of pentane is slowly added to the column. This eluate is also collected and discarded.

C. Chemical class separations

1. The sample extract is introduced to the column just as the pentane rinse level reaches the silica gel. The vial is then rinsed with an additional 1 ml of pentane which is also introduced to the column just before the silica gel is exposed. The eluate is collected in a clean round bottom flask.
2. As the sample rinse level reaches the silica gel, 45 ml of pentane is added to the column. The eluate is collected as the F-1 fraction in a clean Turbo-Vap flask.
3. As the pentane level reaches the top of the silica, 36 ml of a 70:30 (volume:volume) pentane: CH_2Cl_2 mixture is introduced to the column. The F-2 fraction is collected is collected in the same flask as the F-1 fraction. After collection, the flasks are kept tightly capped with aluminum foil. At no time should the column flow rate exceed 6 ml/min.
4. After the F-2 fraction has been collected from the column, the flasks are placed in the Turbo-Vap. The apparatus is turned on and Nitrogen gas is introduced to the flasks. The solvent is reduced to approximately 1 ml. The samples are then solvent-exchanged to heptane and concentrated to about 1 ml.

**ERL-N STANDARD OPERATING PROCEDURE
COLUMN CHROMATOGRAPHY OF SEMIVOLATILE
ORGANIC ANALYTES**

**ERL-N SOP 2.03.005
REVISION 0
JANUARY 1991
PAGE 3 OF 3**

5. The fractions are then transferred to borosilicate glass vials fitted with Teflon-lined screw caps for storage until analysis.

IV. TROUBLE SHOOTING

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
DIGESTION OF ORGANISM SAMPLES FOR TRACE METAL
ANALYSIS**

ERL-N SOP 2.03.006
REVISION 0
JANUARY 1991
PAGE 1 OF 3

POINT OF CONTACT:

Chemistry Group
US Environmental Protection Agency and
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I. OBJECTIVE

This SOP describes the procedure for digestion of organism tissue samples for analysis for trace metals.

II. NECESSARY MATERIALS AND EQUIPMENT

- Stainless steel dissection instruments
- Well-characterized reference materials such as NBS tissue or ERL-N mussel homogenate
- Tared, acid-cleaned, 100- or 250-ml borosilicate glass beakers
- Borosilicate glass watch glasses
- Laboratory scale
- Virtis lyophilizer No.#10-145MR-BA
- HNO₃ (Insta-Analyzed grade), concentrated and 2M
- Acid-washed Whatman 42 filter paper
- Volumetric flask, 50 ml
- Acid-washed polyethylene bottles (60 ml) and vials (20 ml)
- 30% H₂O₂
- Heat source

III. METHOD

A. Sample Preparation

1. Thaw organism samples from each sampling site prior to dissection and separate from shell or skin.
2. Remove tissue specimens for analysis using stainless steel instruments. To protect against cross-contamination, clean instruments between samples by rinsing deionized water.
3. Well-characterized reference materials such as NBS tissue or ERL-N mussel homogenate and procedural blanks should be prepared along with the unknown samples.

**ERL-N STANDARD OPERATING PROCEDURE
DIGESTION OF ORGANISM SAMPLES FOR TRACE METAL
ANALYSIS**

ERL-N SOP 2.03.006
REVISION 0
JANUARY 1991
PAGE 2 OF 3

B. Sample weighing/drying

1. Place portions of each specimen in tared, acid-cleaned, 100- or 250-ml borosilicate glass beakers and cover with borosilicate watch glasses. Obtain the wet weight for each sample.
2. Freeze-dry samples in a Virtis lyophilizer No.#10-145MR-BA for 48 hr. at -40 C and 24 hr. at 30 C. Remove from the dryer and obtain dry weights for the samples.

C. Sample digestion

1. Add 10-20 ml of concentrated HNO_3 (Insta-Analyzed grade) to each sample and digest for 24 hr. at room temperature.
2. Add an additional 20 ml of acid to each sample. Heat the beakers to 60 C and maintain at that temperature until digestion is complete, adding acid as necessary.
3. Remove covers and heat the solutions to evaporate the acid to dryness. Redissolve the residue from each sample with 20 ml of 2M HNO_3 .
4. Filter through acid-washed Whatman 42 filter paper into a 50-ml volumetric flask. Rinse the sample beaker with two 10 ml washes of 2M HNO_3 , which are also filtered and combine with the initial solution.
5. Bring the final sample solution to 50 ml by addition of 2M HNO_3 and store in 60-ml acid-cleaned, polyethylene bottles.

D. Mercury analysis

Tissue samples prepared for mercury analysis undergo a similar procedure, but the samples are not dried prior to acid digestion.

1. Dry weights are calculated from the wet weights measured and the dry-to-wet weight ratios obtained from the corresponding trace metal samples.
2. When the digested sample solution volume is reduced to about 1-2 ml, 5 ml of 30% H_2O_2 is added and the samples heated. This step is necessary to decompose organic matter and surface-active compounds which cause foaming and interferences in the hydride reaction step of the mercury analysis.
3. The samples are evaporated to near-dryness, dissolved in 10 ml of 2N HNO_3 and stored in acid-washed 20-ml polyethylene vials.

IV. TROUBLE SHOOTING

Discussed in methods.

**ERL-N STANDARD OPERATING PROCEDURE
DIGESTION OF ORGANISM SAMPLES FOR TRACE METAL
ANALYSIS**

ERL-N SOP 2.03.006
REVISION 0
JANUARY 1991
PAGE 3 OF 3

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
MICROWAVE DIGESTION OF ORGANISM SAMPLES FOR
INORGANIC ANALYSIS**

ERL-N SOP 2.03.007
REVISION 0
JANUARY 1991
PAGE 1 OF 4

POINT OF CONTACT:

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I. OBJECTIVE

This SOP describes a procedure for complete digestion of organism tissue samples for inorganic analysis by atomic absorption or emission spectrophotometry. The tissue is digested in concentrated nitric acid by a two-step, microwave-assisted heating process. The first (open-vessel) step provides for substantial decomposition and dissolution of the organic tissue matrix; the second (closed-vessel) step utilizes hydrogen peroxide and the higher temperatures and pressures obtained with sealed digestion vessels to complete the digestion of the tissue in the acid, allowing measurement of the associated metals.

II. NECESSARY MATERIALS AND EQUIPMENT

- Stainless steel dissection instruments
- Deionized water
- Tissue homogenizing system (not stainless steel if chromium and/or nickel are to be analyzed)
- Teflon digestion vessels with peel-off labels
- Laboratory scale
- Freezer
- Virtis lyophilizer
- HNO₃ (Instra-Analyzed grade), concentrated and 2M
- MDS-81
- H₂O₂
- Plastic tweezers
- Whatman 42 filter paper
- Filtration apparatus
- Acid-cleaned polyethylene bottle, 60 ml
- Volumetric flask, 50 ml

III. METHOD

A. Sample preparation

1. Organism samples should be thawed prior to dissection. Removed tissue specimens from shell or skin using stainless steel instruments. Rinse instruments between samples

**ERL-N STANDARD OPERATING PROCEDURE
MICROWAVE DIGESTION OF ORGANISM SAMPLES FOR
INORGANIC ANALYSIS**

ERL-N SOP 2.03.007
REVISION 0
JANUARY 1991
PAGE 2 OF 4

by with deionized water. If required, homogenize samples using appropriate tissue homogenizing system (do not use stainless steel generators if chromium and/or nickel are to be analyzed).

2. Number the empty Teflon digestion vessels with peel-off labels and obtain the tare weight of each vessel (without the pressure relief disk).

3. Add approximately 15 g of wet tissue (approximately 2.5 g dry tissue) to each vessels and reweigh, obtaining the wet gross weight. Place the vessels upright in freezer until specimens are frozen solid.

4. Freeze-dry the samples using the Virtis lyophilizer.

a. Drain the condenser, then pre-cool to -50 C. Refrigerate sample compartment below 0 C.

b. Place frozen specimens in sample compartment. Seal door, close vacuum release clamp and start vacuum pump. Verify that vacuum is being drawn (pressure < 1.5 torr).

c. Freeze-dry specimens for 48 hr. at -40 C, then turn on shelf heat and hold for 24 hr at 45 C.

5. Remove the vessels from the freeze dryer and weigh again, obtaining the dry gross weights for the samples.

B. Microwave digestion

1. Open-vessel digestion

a. Add 15 ml of concentrated HNO₃ (Instra-Analyzed grade) to each sample vessel and close cap, without pressure relief disks, hand-tight. If bubbling or foaming occurs, allow samples to sit at room temperature until foaming subsides (1 hr.).

b. Load vessels into carousel, place carousel into microwave oven and close door. Begin carousel rotation, making sure oven exhaust fan is operating.

c. Program MDS-81:

	Time(min)	6 vessels	Power level	
			8 vessels	12 vessels
S-1:	3:00	25 %	30 %	35 %
S-2:	5:00	35 %	40 %	55 %
S-3:	5:00	50 %	60 %	75 %

and press START to initiate microwave digestion.

d. After program has completed run, remove sample carousel from MDS-81 and

**ERL-N STANDARD OPERATING PROCEDURE
MICROWAVE DIGESTION OF ORGANISM SAMPLES FOR
INORGANIC ANALYSIS**

ERL-N SOP 2.03.007
REVISION 0
JANUARY 1991
PAGE 3 OF 4

place in hood to cool.

2. Closed vessel digestion

a. Remove cap from each vessel and add 3 ml H_2O_2 to vessel. Place pressure relief disk, ring side up, on top of lower portion of vessel and replace cap hand-tight. Tighten caps to correct torque using MDS-81 capping station.

b. Place vessels in carousel. Insert vent tube into each vessel neck and tighten nut. Insert free end of tube into vent trap in center of carousel and return carousel to oven. Insure that venting fan is operating and begin carousel rotation.

c. Program MDS-81:

			Power Level		
	Time(min)	6 vessels	8 vessels	12 vessels	
S-1:	6:00	40%	50%	60%	
S-2:	2:00	0%	0%	0%	
S-3:	5:00	50%	60%	80%	

and press START to initiate microwave digestion.

d. After program is completed, remove carousel from MDS-81 and place in hood to cool (minimum 1 hour). When vessels are cool to touch, remove vent tubes and CAREFULLY vent vessels manually to release pressure. If venting is too vigorous, allow to cool longer and vent again. Repeat until no more venting occurs.

e. Remove caps from vessels using MDS-81 capping station. Invert cap and pressure relief disk over vessel and rinse with deionized water, allowing rinse to drain into vessel. Add 15 ml of deionized water to vessel.

C. Sample filtration and dilution

1. Using plastic tweezers, place circle of Whatman 42 filter paper into filtration apparatus. Wash filter with 2 M HNO_3 . Place 60-ml acid-cleaned, polyethylene bottle and vacuum gasket under filtration apparatus and apply vacuum. Filter digested sample solution through filter paper into bottle. Rinse the digestion vessel with deionized water and pour through filter as well. Repeat rinse/filtration. Holding sample bottle, release vacuum and remove bottle.

2. Pour combined filtrates from bottle into 50 ml volumetric flask. Rinse bottle and use the rinse to dilute solution in flask to the volume mark. Discard any remaining rinse solution in bottle. Return the sample solution to the bottle and label bottle appropriately.

IV. TROUBLE SHOOTING

Discussed in methods.

**ERL-N STANDARD OPERATING PROCEDURE
MICROWAVE DIGESTION OF ORGANISM SAMPLES FOR
INORGANIC ANALYSIS**

**ERL-N SOP 2.03.007
REVISION 0
JANUARY 1991
PAGE 4 OF 4**

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
PREPARATION OF WATER SAMPLES FOR DIRECT
DETERMINATION OF TRACE METALS**

ERL-N SOP 2.03.008
REVISION 0
FEBRUARY 1991
PAGE 1 OF 2

POINT OF CONTACT:

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I. OBJECTIVE

This SOP describes the preparation of water samples (saline or fresh) for direct determination of trace metals by atomic absorption spectrophotometry. The preparation procedure is the same determination of either soluble or total metals; if analysis of soluble metals is desired, samples should be filtered prior to acidification. Acidification with nitric acid liberates metals from any suspended particulate matter in the sample (except for resuspended sediment minerals) and prevents adsorption of the dissolved metals to the container walls.

II. NECESSARY MATERIALS AND EQUIPMENT

- Acid-cleaned polyethylene bottles
- HNO₃ (Seastar brand or equivalent), concentrated
- pH meter
- Pipette, 1 ml
- Acid-cleaned polyethylene vials
- Chelex-100
- Clean seawater or NASS-1 open ocean reference seawater

III. METHOD

1. Samples should be collected in acid cleaned polyethylene bottles; if soluble and particulate metals are to be determined, filtration should be performed as soon after sample collection as possible.
2. Samples should be acidified to a pH of approximately 2-2.5 with 1 microliter of conc. HNO₃ (Seastar brand or equivalent) per milliliter of sample. Samples should sit for at least 1 hour before proceeding in order to completely recover particulate metals and metals adsorbed to the container walls (although ultrasonic agitation may reduce the amount of time necessary). Once acidified, the samples can be stored for long periods of time before analysis.
3. Pipette one milliliter (1 ml) of each sample into acid cleaned polyethylene vials. Add 100 microliters of conc. HNO₃ (Seastar brand or equivalent), close the vial and shake well.

**ERL-N STANDARD OPERATING PROCEDURE
PREPARATION OF WATER SAMPLES FOR DIRECT
DETERMINATION OF TRACE METALS**

**ERL-N SOP 2.03.008
REVISION 0
FEBRUARY 1991
PAGE 2 OF 2**

4. Standards should be prepared from 1 milliliter of Chelex-100 stripped seawater or the NASS-1 open ocean reference seawater and 100 microliters of conc. HNO_3 (Seastar brand or equivalent).

IV. TROUBLE SHOOTING

Discussed in methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF SEAWATER SAMPLES FOR
ORGANIC ANALYSIS**

ERL-N SOP 2.03.009

REVISION 0

JANUARY 1991

PAGE 1 OF 2

POINT OF CONTACT:

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I. OBJECTIVE

This method is used to prepare water column samples for column fractionation.

II. NECESSARY MATERIALS AND EQUIPMENT

- 1 L water sample
- Separatory funnel, 2 L
- Methylene chloride
- Graduated cylinders and volumetric flasks, assorted sizes
- Recovery standards
- Erlenmeyer flask, 500 mL
- Anhydrous sodium sulfate
- Round bottom flask
- K-D flask
- Snyder column
- Heating mantle
- Hexane
- Graduated concentrator tube
- Microsnyder column
- Ebulator
- Standard laboratory safety equipment

III. METHOD

1. The 1 L sample is transferred from a sample bottle to a 2 liter separatory funnel. The inside of the bottle is rinsed 3X with the first 50mls of methylene chloride and the rinses are added to the separatory funnel.
2. Add the recovery standards.
3. The sample is extracted 2X with 50 mL of methylene chloride and the extracts are collected in a 500ml Erlenmeyer flask.
4. Add anhydrous sodium sulfate to the combined extracts to remove any water.

**ERL-N STANDARD OPERATING PROCEDURE
EXTRACTION OF SEAWATER SAMPLES FOR
ORGANIC ANALYSIS**

**ERL-N SOP 2.03.009
REVISION 0
JANUARY 1991
PAGE 2 OF 2**

5. Transfer the sample to a round bottom flask and rinse the sodium sulfate 3X with methylene chloride. The round bottom is then fitted with a K- D flask and snyder column and placed on a heating mantle. The solvent is volume reduced and solvent exchanged to hexane.

6. Further reduce the volume to less than 1ml in a graduated concentrator tube fitted with a microsnyder column and an ebulator. Cool and make the final volume to 1ml with hexane.

7. The sample is then fractionated as outlined in column fractionation procedure.

IV. TROUBLE SHOOTING

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
SEDIMENT EXTRACTION FOR SEMIVOLATILE ORGANIC
ANALYTES**

**ERL-N SOP 2.03.010
REVISION 0
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PAGE 1 OF 3**

POINT OF CONTACT:

Chemistry Group
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I. OBJECTIVES

The objective of this document is to define the standard operating procedure for the extraction of semi-volatile organic compounds from marine sediment samples. The extracts will be further cleaned up by silica gel chromatography procedures prior to analysis by gas chromatography (GC) or gas chromatography/mass spectrometry (GC/MS).

II. NECESSARY MATERIALS AND EQUIPMENT

- Ultrasonic probe
- 100- or 150-ml stainless steel centrifuge tubes
- Top-loading balance capable of weighing to 0.01 g
- Aluminum weighing pans
- Stainless steel spatula
- Drying oven maintained at 105-120°C
- Turbo-Vap (Zymark) apparatus, with heated water bath maintained at 25-35° C
- Nitrogen gas, compressed, 99.9% pure
- Glass Turbo-Vap flasks, 200 ml
- Glass graduated cylinders, 100- and 500-ml
- Glass separatory funnels, 1 l.
- Erlenmeyer flasks, 250 and 500 ml.
- Microliter syringes or micropipets, solvent rinsed
- Borosilicate glass vials with Teflon-lined screw caps, 2-ml
- Reagents
 - Pentane, pesticide grade or equivalent
 - Acetonitrile, pesticide grade or equivalent
 - Deionized water, pentane-extracted
 - Acetone, pesticide grade or equivalent
 - Sodium sulfate-anhydrous, reagent grade. Heated to 400°C for at least 4 hours, then cooled and stored in a tightly-sealed glass container at room temperature.
 - Internal Standards, to be added to each sample prior to extraction.

III. METHODS

1. Weigh approximately 10.0g of homogenized sample into a solvent rinsed

**ERL-N STANDARD OPERATING PROCEDURE
SEDIMENT EXTRACTION FOR SEMIVOLATILE ORGANIC
ANALYTES**

ERL-N SOP 2.03.010
REVISION 0
JANUARY 1991
PAGE 2 OF 3

(acetone/pentane) centrifuge tube. Homogenization is accomplished by physical mixing of the sediment with stainless steel or Teflon coated utensils, or by a polyethylene propeller attached to an electric drill. The amount of sample may be adjusted based on expected contaminant concentrations or detection limits required. Weigh approximately 2.0 grams into a preweighed aluminum pan for dry/wet determination.

2. Add Internal Standards as required: OCN for PCB Analysis; Gamma Chlordene for Pesticides; and d12 Benzo(a)Anthracene and d10 Phenanthrene mix for PAHs. The amount of IS added is dependent on the expected contaminant concentrations and should be equivalent to those concentrations.

3. Add 50 ml acetonitrile.

4. Sonicate the samples for 60 seconds, at a speed setting of ~ 5. Centrifuge for 10 minutes at 1750 rpm and pour off the supernatant into a separatory funnel containing 300ml pentane extract and deionized water (DI). Repeat this step three more times, each with 50 ml acetonitrile.

5. Back extract the DI/ACETONITRILE phase in the separatory funnel with 3X 50ml pentane. After each addition of pentane has been shaken, draw off the bottom layer into a 500ml erlenmeyer flask. Decant the Pentane layer into a 250ml erlenmeyer flask by pouring it out the top of the separatory funnel. This way the transfer of water into the pentane extract will be avoided.

6. Transfer the water layer from the 500 ml erlenmeyer flask back into the separatory funnel for every addition of pentane. Rinse the 500ml flask three times with Pentane and add the rinses to the separatory funnel.

7. Combine the pentane extracts and dry over Sodium Sulfate.

8. Transfer the sample to a 200 ml Turbo-Vap flask. Rinse the flask 3x with pentane and add the rinses to the flask. Place the flask into the Turbo-Vap apparatus, and turn on the unit. Open the valve on the nitrogen tank and set the regulator to ensure a pressure of 15 psig is reaching the Turbo-Vap unit. Reduce the volume of sample to approximately 1 ml.

9. Adjust the volume to 1 ml with pentane. Transfer the sample to a borosilicate vial.

10. Fractionate the sample following the Column Chromatography SOP.

IV. TROUBLE SHOOTING

1. Activated copper powder (activated by the addition of 8 M hydrochloric acid and rinsed with the following solvents in succession: deionized water, methanol, methylene chloride, and hexane) may be added to the extract to remove any free elemental sulfur. The copper is added until the formation of black copper sulfide no longer occurs.

**ERL-N STANDARD OPERATING PROCEDURE
SEDIMENT EXTRACTION FOR SEMIVOLATILE ORGANIC
ANALYTES**

**ERL-N SOP 2.03.010
REVISION 0
JANUARY 1991
PAGE 3 OF 3**

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
TISSUE EXTRACTION FOR SEMIVOLATILE ORGANIC
ANALYTES**

ERL-N SOP 2.03.011
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PAGE 1 OF 2

POINT OF CONTACT:

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I. OBJECTIVES

The objective of this document is to define the standard operating procedure for the extraction of semi-volatile organic compounds from marine tissue samples. The extracts will be further cleaned up by silica gel chromatography procedures prior to analysis by gas chromatography (GC) or gas chromatography/mass spectrometry (GC/MS).

II. MATERIALS AND EQUIPMENT

- Brinkman Polytron
- 100- or 150-ml glass centrifuge tubes
- Top-loading balance capable of weighing to 0.01 g
- Aluminum weighing pans
- Stainless steel spatula
- Drying oven maintained at 105-120°C
- Turbo-Vap (Zymark) apparatus, with heated water bath maintained at 25-35° C
- Nitrogen gas, compressed, 99.9% pure
- Glass Turbo-vap flasks, 200 ml
- Glass graduated cylinders, 100- and 500-ml
- Glass separatory funnels, 1 L.
- Glass erlenmeyer flasks, 250 and 500 ml.
- Borosilicate glass vials with Teflon-lined screw caps, 2-ml
- Microliter syringes or micropipets, solvent rinsed
- Reagents
 - Pentane, pesticide grade or equivalent
 - Acetonitrile, pesticide grade or equivalent
 - Deionized water, pentane-extracted
 - Sodium sulfate-anhydrous, reagent grade. Heated to 400°C for at least 4 hours, then cooled and stored in a tightly-sealed glass container at room temperature.
 - Internal Standards, to be added to each sample prior to extraction.

III. METHODS

1. Weigh approximately 10.0g of sample into a solvent rinsed centrifuge tube. Weigh approximately 1.0 gram into a preweighed aluminum pan for dry/wet determination.

2. Add Internal Standards as required: OCN for PCB Analysis; Gamma Chlordene for Pesticides; and d12 Benzo(a)Anthracene and d10 Phenanthrene mix for PAHs. The amount of IS added is dependent on the expected contaminant concentrations and should be equivalent to those concentrations.

3. Add 50 ml acetonitrile.

4. Polytron the samples for 20 seconds, at a speed setting of ~ 5. Centrifuge for 10 minutes at 1750 rpm and pour off the supernatant into a separatory funnel containing 500ml pentane extracted deionized water (DI). Repeat this step two more times.

5. Back extract the DI/ACETONITRILE phase in the separatory funnel with 3X 50ml pentane. After each addition of pentane has been shaken, draw off the bottom layer into a 500ml erlenmeyer flask. Decant the Pentane layer into a 250ml erlenmeyer flask by pouring it out the top of the separatory funnel. This way the transfer of water into the pentane extract will be avoided.

6. Transfer the water layer from the 500 ml erlenmeyer flask back into the separatory funnel for every addition of pentane. Rinse the 500ml flask 3 x with Pentane and add the rinses to the separatory funnel.

7. Combine the pentane extracts and dry over Sodium Sulfate.

8. Transfer the sample to a 200 ml Turbo-Vap flask. Rinse the flask 3x with pentane and add the rinses to the flask. Place the flask into the Turbo-Vap apparatus, and turn on the unit. Open the valve on the nitrogen tank and set the regulator to ensure a pressure of 15 psig is reaching the Turbo-Vap unit. Reduce the volume of sample to approximately 1 ml.

9. Adjust the volume to 1.0 ml with pentane. Remove 0.1ml of sample into a preweighed aluminum pan for lipid weight determination. Allow it to dry at room temperature for at least 24 hours. Record the weight of the pan plus the sample.

10. Fractionate the sample following the Column Chromatography SOP.

IV. TROUBLE SHOOTING

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
TOTAL MICROWAVE DIGESTION OF SEDIMENT SAMPLES
FOR INORGANIC ANALYSIS**

ERL-N SOP 2.03.012
REVISION 0
JANUARY 1991
PAGE 1 OF 4

POINT OF CONTACT:

Chemistry Group
US Environmental Protection Agency and
Science Applications International Corporation
c/o US Environmental Protection Agency
27 Tarzwell Dr.
Narragansett, RI 02882

I. OBJECTIVE

This SOP describes the procedure for complete digestion of sediments for determination of metals concentrations. Because of the digestion is complete, the concentrations measured are the total metals concentrations in the sediment, including both anthropogenic and natural background components.

II. NECESSARY MATERIALS AND EQUIPMENT

- Concentrated nitric acid
- Hydrofluoric acid
- Boric acid
- Teflon digestion vessels with peel-off labels
- Freezer
- Virtis lyophilizer
- Laboratory scale
- Protective clothing:
 - Labcoat
 - Polyethylene apron
 - Neoprene gloves
 - Safety goggles (not glasses)
 - Face shield
- MDS-81
- Fume hood
- 50 ml volumetric flask
- Deionized water
- Clean, acid-stripped polyethylene bottle

III. METHOD

Complete digestion of the mineral matrix is accomplished by the use of concentrated nitric and hydrofluoric acid, which must be neutralized by reaction with boric acid prior to analysis in order to prevent etching of glassware in the analytical instrumentation.

A. Sample preparation

**ERL-N STANDARD OPERATING PROCEDURE
TOTAL MICROWAVE DIGESTION OF SEDIMENT SAMPLES
FOR INORGANIC ANALYSIS**

ERL-N SOP 2.03.012
REVISION 0
JANUARY 1991
PAGE 2 OF 4

1. Sediments should be thawed and homogenized using appropriate equipment prior to subsampling for analysis.
2. Number the empty Teflon digestion vessels with peel-off labels and obtain the tare weight of each vessel (without the pressure relief disk).
3. Add approximately 1.0-1.5 g of wet sediment (0.5-0.75 g dry) to each vessel and reweigh, obtaining the wet gross weight. Place the vessels upright in freezer until the sediments are frozen solid.
4. Freeze-dry the samples using the Virtis lyophilizer.
 - a. Drain the condenser, then pre-cool to -50 C. Refrigerate sample compartment below 0 C.
 - b. Place frozen specimens in sample compartment. Seal door, close vacuum release clamp and start vacuum pump. Verify that vacuum is being drawn (pressure < 1.5 torr).
 - c. Freeze-dry specimens for 48 hr. at -40 C, then turn on shelf heat and hold for 24 hr at 45 C.
5. Remove the vessels from the freeze dryer and weigh again, obtaining the dry gross weights for the samples.

B. Microwave digestion

1. Before digesting the sediment samples, the chemist must be wearing appropriate protective clothing: labcoat, polyethylene apron, neoprene gloves, safety goggles (not glasses) and face shield.
2. Add 3.0 ml of concentrated nitric acid (HNO_3) to each sample vessel. Swirl slightly to wet sediment and check for reaction with sediment, e.g. foaming or bubbling. When no reaction is evident, add 3.0 ml of concentrated hydrofluoric acid (HF) to each sample vessel. Place pressure relief disk, ring side up, on top of lower portion of vessel and replace cap hand-tight. Tighten caps to correct torque using MDS-81 capping station.
3. Place vessels in carousel. Insert vent tube into each vessel neck and tighten nut. Insert free end of tube into vent trap in center of carousel and return carousel to oven. Insure that venting fan is operating and begin carousel rotation.
4. Program MDS-81:

# of vessels =	4	6	8
Step	Time	Power (%)	
s-1:	3:00	40	55 68

**ERL-N STANDARD OPERATING PROCEDURE
TOTAL MICROWAVE DIGESTION OF SEDIMENT SAMPLES
FOR INORGANIC ANALYSIS**

ERL-N SOP 2.03.012
REVISION 0
JANUARY 1991
PAGE 3 OF 4

s-2:	6:00	75	100	100* (* digest for 7:30)
s-3:	20:00	45	60	75

and press START to initiate microwave digestion.

5. After program is completed, remove carousel from MDS-81 and place in hood to cool (minimum 30 minutes). When vessels are cool to touch, remove vent tubes and CAREFULLY vent vessels manually to release pressure. If venting is too vigorous, allow to cool longer and vent again. Repeat until no more venting occurs.

6. Remove caps from vessels using MDS-81 capping station. Add 30 ml of 5% boric acid solution to each vessel, replace pressure relief disk and cap, and torque, using MDS-81 capping station, as above.

7. Program MDS-81:

# of vessels =	4	6	8
Step	Time	Power (%)	
s-1:	15:00	45	60 75

and press START.

8. After program is completed, remove carousel from MDS-81 and place in hood to cool. When vessels are cool, remove vent tubes and vent vessels. Observe same precautions as above.

C. Sample filtration and dilution

1. Transfer contents of each digestion vessel without filtering into a 50-ml volumetric flask.
2. Rinse the vessel with deionized water, adding the rinse to the volumetric flask. Dilute with deionized water to the volumetric mark.
3. Pour the sample solution into a clean, acid-stripped polyethylene bottle and label the bottle appropriately.

IV. TROUBLE SHOOTING

Discussed in methods section.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

**ERL-N STANDARD OPERATING PROCEDURE
TOTAL MICROWAVE DIGESTION OF SEDIMENT SAMPLES
FOR INORGANIC ANALYSIS**

ERL-N SOP 2.03.012
REVISION 0
JANUARY 1991
PAGE 4 OF 4

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
ULTRASONIC EXTRACTION OF SEDIMENT SAMPLES
FOR INORGANIC ANALYSIS**

**ERL-N SOP 2.03.013
REVISION 0
FEBRUARY 1991
PAGE 1 OF 3**

POINT OF CONTACT:

**Chemistry Group
US Environmental Protection Agency and
Science Applications International Corporation
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27 Tarzwell Dr.
Narragansett, RI 02882**

I. OBJECTIVE

This SOP describes the method through which trace metals are extracted from sediments utilizing 2M HNO₃ and ultrasonic agitation. The extracted metals are then separated from the sediment residue by centrifugation. The resultant solution is used for trace metals analysis by flame or graphite furnace atomic absorption or inductively coupled plasma emission spectrometry. This procedure will dissolve metals present in the sediment in most chemical forms, but does not attack the sediment mineral matrix; consequently, the concentrations measured provide an estimate of the maximum environmentally available metals concentrations, rather than the total concentrations of metals in the sediment.

II. NECESSARY MATERIALS AND EQUIPMENT

- Dry, acid-washed polycarbonate centrifuge tubes with caps and peel-off labels
- Laboratory scale
- Freezer
- Virtis lyophilizer (freeze-dryer)
- 2M HNO₃
- Ultrasonic bath
- Centrifuge
- Volumetric flask, 50 ml
- Acid-stripped 60-ml polyethylene bottle

III. METHOD

A. Sample preparation

1. Samples should be thawed and homogenized prior to subsampling for analysis.
2. Label or number dry, acid-washed polycarbonate centrifuge tubes, e.g. with peel-off labels, and obtain the tare weight of each tube.
3. Add approximately 5 g of wet sediment (2 - 2.5 g dry) to each tube and reweigh, obtaining the wet gross weight. Place the tubes upright in freezer until sediments are frozen solid.

**ERL-N STANDARD OPERATING PROCEDURE
ULTRASONIC EXTRACTION OF SEDIMENT SAMPLES
FOR INORGANIC ANALYSIS**

ERL-N SOP 2.03.013
REVISION 0
FEBRUARY 1991
PAGE 2 OF 3

4. Freeze-dry the samples using the Virtis lyophilizer (freeze-dryer).

- a. Drain the condensor, then precool to -50 C. Refrigerate sample compartment below 0 C.
- b. Remove the frozen specimens from the freezer. Open the centrifuge caps 3/4-1 full turn. Place the frozen specimens in sample compartment of the freeze-dryer. Seal door, close vacuum release clamp and start vacuum pump. After 15 minutes, verify that vacuum is being drawn (pressure < 1.5 torr).
- c. Freeze-dry specimens for 16-24 hr. at -40 C.
- d. Release vacuum and ensure that no standing water remains in the tube above the sediment; if there is standing water, continue freeze-drying at -40 C for another 24 hr.
- e. When Standing water is no longer evident, turn on shelf heat and hold for 24 hr at 45 C and < 1.5 torr.

5. Remove the tubes from the freeze dryer and weigh again, obtaining the dry gross weights for the samples.

B. Ultrasonic Extraction

1. Add approximately 25 ml of 2M HNO₃ to each sample tube and replace cap hand-tight.
2. Place tubes in ultrasonic bath rack. Insure that all tubes are completely vertical and contained within openings on bottom of rack. Place cover over rack and fasten, using nylon wing nuts, hand-tight. Place covered rack into ultrasonic bath and insure that bath level is at or above level of acid in the tubes.
3. Turn on ultrasonic bath and ultrasonicate samples approximately 16 hours (overnight). Bath will heat to about 75 C.
4. Remove rack and tubes from ultrasonic bath, allow to drain and cool.
5. Weigh centrifuge tubes. Pair tubes as closely as possible by gross weight. Centrifuge at 10,000 rpm for 15 minutes. Decant supernatant from tube into 50 ml volumetric flask.
6. Add 15 ml of 2M HNO₃ to residue in tube and ultrasonicate for another 1 hour. Repeat centrifugation as in step 5 and decant into flask.
7. Dilute solution in flask to the volume mark with 2M HNO₃. Pour solution into acid-stripped 60-ml polyethylene bottle and label bottle appropriately (sample ID and REP, etc.).

**ERL-N STANDARD OPERATING PROCEDURE
ULTRASONIC EXTRACTION OF SEDIMENT SAMPLES
FOR INORGANIC ANALYSIS**

**ERL-N SOP 2.03.013
REVISION 0
FEBRUARY 1991
PAGE 3 OF 3**

IV. TROUBLE SHOOTING

Discussed in methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
ANALYSIS OF DISSOLVED PCBS USING FOAM PLUGS**

**ERL-N SOP 2.04.001
REVISION 0
JANUARY 1991
PAGE 1 OF 3**

POINT OF CONTACT:

**Chemistry Group
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Narragansett, RI 02882**

I. OBJECTIVE

The objective of this document is to describe the procedure for the preparation and extraction of foam plugs when they are used to measure dissolved polychlorinated biphenyls in seawater.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware*

- 1 liter separatory funnels
- 500 and 250 ml Erlenmeyer flasks
- 100 ml graduated cylinder
- Kuderna-Danish flasks
- Snyder columns
- 500 ml round bottom flasks
- graduated concentrator tubes
- microsnyder columns
- ebulators
- 2 ml screw top vials
- 100 μ l syringe
- pasteur pipets
- boiling beads
- glass rod - 0.5" diameter
- * all glassware were combusted at 450°C for 6 hours

B. Chemicals

- Acetone - high purity grade
- Hexane UV - pesticide grade
- Sulfuric acid -concentrated
- Sodium sulfate - anhydrous baked at 750°C for 4 hours
- foam plugs - IDENTI-PLUG size D 35-45 mm
- teflon foam plug housings w/closures - 10 inch length
- heating mantle for round bottom flasks
- concentrator tube heater

III. METHOD

A. Clean-up Procedure

1. Wash foam plug housings and end closures with Alconox and water. Thoroughly rinse with DI water. Let dry.
2. Push three foam plugs down to one end of the housing with a glass rod. Be careful to not distort the shape of the plug or to push them together too hard. They should be just touching. The flow through the housing will be toward the plugs. Label the housing to show the direction of the flow.
3. Attach the closure at the plugged end of the housing, remove the nut and secure the housing in the hood with the plugged end down. Add solvents in the following order:

- 2 x 50 ml acetone
- 2 x 50 ml hexane
- 2 x 50 ml acetone
- 4 x 50 ml DI water

Wait for each solvent to drain through the housing before adding the next one.

4. Replace the nut on the plugged end of the housing and add the closure at the other end of the housing. Wrap in aluminum foil.

B. Extraction Procedure

1. Wipe any excess water from the outside of the foam plug housing. Secure the housing in the hood in such a way that the plugged end is down and positioned over a separatory funnel containing 200 mls of hexane extracted DI water. It is helpful to position a hexane rinsed glass funnel in the top of the separatory funnel. Remove the nut from the plugged end of the housing and remove the entire closure from the other end.
2. Add the solvents to the housing in the following order, allowing each to drain into the separatory funnel before adding the next. In between solvent additions, use a clean glass rod to squeeze the solvents through the plugs.
 - 2 x 50 ml acetone
 - 2 x 50 ml hexane
3. Add the appropriate amount of OCN internal standard to each separatory funnel.
4. Shake the separatory funnel, allow the layers to separate and draw off the bottom

**ERL-N STANDARD OPERATING PROCEDURE
ANALYSIS OF DISSOLVED PCBS USING FOAM PLUGS**

**ERL-N SOP 2.04.001
REVISION 0
JANUARY 1991
PAGE 3 OF 3**

aqueous phase into a clean 500 ml Erlenmeyer flask. Pour off the hexane layer into a clean 250 ml Erlenmeyer flask. Return the aqueous phase to the separatory funnel and extract it again with 100 ml of hexane. Combine the second hexane extract with the first.

5. Add anhydrous sodium sulfate to the hexane extracts.
6. Transfer the hexane into a 500 ml round bottom flask, making sure to rinse the sodium sulfate 3X with hexane. The round bottom is then fitted with a K-D and snyder column and placed on a heating mantle. Reduce the volume to approximately 10 mls.
7. Transfer the sample using a pasteur pipet to a 10 ml graduated concentrator tube, fitted with a microsnyder column and an ebulator, and further reduce the volume to 0.5 mls.
8. Bring the volume to 1 ml with hexane and transfer to a screw-top vial to which is added approximately 0.5 ml of concentrated sulfuric acid. The vial is rolled on the lab bench very slowly for one minute. The acid and the sample are allowed to interact for at least 15 minutes.
9. The hexane layer is drawn off from the acid using a pasteur pipet and stored in a second screw top vial.

IV. TROUBLE SHOOTING

1. It is very important to wipe the outside of the foam plug prior to the extraction step. Due to the fact that the plug is stored frozen, condensation collects on the outside and can contaminate the sample.
2. When the sample is rolled with the acid, it should be done slowly. Any vigorous mixing will result in an emulsion.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
GAS CHROMATOGRAPHY-MASS SPECTOMETRY**

ERL-N SOP 2.04.002
REVISION 0
FEBRUARY 1991
PAGE 1 OF 5

POINT OF CONTACT:

Chemistry Group
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I. OBJECTIVE

The objective of this document is to define the standard procedure for analyzing marine environmental samples using GCMS in electron impact/positive ion mode.

II. NECESSARY MATERIALS AND EQUIPMENT

- Finnigan MAT 4530 GCMS system with PPINICI
- Data General Nova 3 computer and Control Data CMD disk drive utilizing Incos 5.5 software

III. METHOD

A. Daily Quality Assurance / System Checks

The following series of system checks is performed at least once a day, i.e. at least once during every 12 hour period of instrument operation. Each check has a previously established acceptable range that can be found in the GCMS logbook. When values are not acceptable, the operator records those values in the logbook and proceeds to make adjustments to correct the condition before continuing with system checks. After the correction has been made, the new values are recorded.

1. Check interface (separator oven), transfer line and manifold temperatures.
2. Check source vacuum (at GC oven temperature 60) and GC column pressure.
3. Establish zero setting using the procedure ZDPLAY. Check instrument sensitivity by admitting perfluorotributylamine (FC-43) to the detector at a pre-determined rate. Record the electron multiplier voltage required to give a 1.6 volt signal at m/e 219 with SENS set at 10**-8. Check peak resolution at low (69/70), mid (219/220) and high (414 or 502) masses. Check the ratios of the peak heights (low to mid, mid to high, etc.). If peak resolution or height ratio adjustments are necessary, record all changes in source tuning in the logbook. Calibrate the instrument using the procedure CALI. Acceptable ranges are Projection Error -150 to +150 MMU, RMS Fit Error < 50 MMU (or < 5% peak width), and Elimination Factor (S.D.) RMS < 10. Make a hard copy and file

it under CALIBRATIONS.

4. Inject 1 ul of decaflourotriphenylphosphine [DFTPP - the EPA GCMS Analytical Quality Control (AQC) compound] at a concentration of 40 ng/ul. Compare fragment peak intensity and relative abundance to the Key Ion Abundance Criteria established by EPA for DFTPP. If unacceptable, repeat system checks beginning at "D" above. Create hard copies of Mass Spectrum and Mass List of DFTPP and file with raw data reports. Check intensity of air peak (28) and column bleed (207).

5. Inject appropriate analytical standard, based on analysis to be performed. Check early, middle and late eluting compounds for peak shape. Choose two compounds with the same mass and strong molecular ions that elute within 8 scans of each other and check resolution.

B. Identification Criteria

1. Identification of peaks run full scan is established by retention times and Extracted Ion Current Profiles (EICP). Compounds must elute within three scans of expected time. If an EICP for a group of related compounds differs markedly from what is expected (based on typical EICP templates), the spectrum of the peak in question is checked to determine it's identity.

2. Identification of peaks run using the multiple ion detection (MID) mode is established by retention times and EICP as above. Due to the lack of a full spectrum, final confirmation is made by monitoring ion masses which are characteristic of the compound in question. Characteristic ion ratios must match standards to within 20% before making final identification.

C. Quantitation Criteria

1. All standards are detailed in the Standards Logbook, which contains information about sources of compounds and methods used to prepare the standards.

D. Internal Standard(s) Quantitation / Use of Authentic Standard

1. Co-inject the authentic standard and the internal standard to determine both of the RFs. Record in the file labelled RESPONSE FACTORS.

2. Measure the ion peak area of the compound and of the internal standard in the sample run.

3. Calculate the concentration of the compound in the original matrix, based on the ratio of the RFs, the measured areas, the amount of internal standard added to the extract, and the dry weight or volume of the sample (see calculation 1).

4. Record the results either in the individual file for the sample, or in the file grouping the results from a number of similar samples.

E. Internal Standard(s) Quantitation / Use of a Similar Compound as a Standard

A similar compound is one (or more) which elutes within a few minutes of the compound of interest, belongs to the same chemical class, and has a key fragment ion within 50 amu of that of the compound of interest.

1. Co-inject the similar compound and the internal standard to determine both of the RFs. Record in the file labelled RESPONSE FACTORS.
2. Measure the ion peak area of the compound and of the internal standard in the sample run.
3. Determine the fragment ion percentages (FIPs) for the key ions in both the compound of interest and the similar standard.
4. Assume the Total Ion Current Response Factors (RFTs) are the same for the similar compound and the standard.
5. Calculate the concentration of the compound in the original matrix, based on the ratios of the internal standard response factors (RFIs) and FIPs., the measured areas, the amount of internal standard added to the extract, and the dry weight or volume of the sample (see calculation 2).
6. Record the results either in the individual file for the sample or in the file grouping the results from a number of similar samples.

F. Internal Standard Quantitation / No Similar Compound is Available for Use as a Standard

1. Choose the closest eluting internal standard which has a key ion(s) similar in mass to a key ion(s) in the compound of interest in the sample.
2. Measure the ion peak area of the compound and of the internal standard in the sample run.
3. Determine the FIPs for the key ions in both the compound of interest and the internal standard.
4. Assume the RFTs are the same for these two compounds.
5. Calculate the concentration of the compound in the original matrix based on the ratio of the FIPs, the measured areas, the amount of internal standard added to the extract, and the dry weight or volume of the sample (see calculation 3).
6. Record the results either in the individual file for the sample or in the file grouping the results from a number of similar samples.

G. Calculations

1. $CONC = AREAC * RFI * NGI / (AREAI * DWORVOL * RFC)$

AREAC = area counts of the quantitation ion of the compound of interest
AREAI = area counts of the quantitation ion of the internal standard
CONC = concentration of the compound of interest in the sample
= nanograms/(gm dry weight) for organisms and sediments
= parts per trillion for water samples
DWORVOL = dry weight (grams) of volume (liters) of sample
RFI = response factor of the internal standard
RFC = response factor of the compound of interest
NGI = nanograms of internal standard added to the extract

2. $CONC = AREAC * RFI * NGI / (AREAI * DWORVOL * RFS * FIPC / FIPS)$

RFS = response factor of the similar compound
FIPC = fragment ion percentage of the quantitation ion in the spectrum of the compound of interest.
FIPS = fragment ion percentage of the quantitation ion in the spectrum of the similar compound

3. $CONC = AREAC * FIPI * NGI / (AREAI * FIPS * DWORVOL)$

FIPI = fragment ion percentage of the quantitation ion in the spectrum of the internal standard

Table 1. Reference Compound Key Ions and Ion Abundance Criteria.

Mass	Ion Abundance Criteria
51	30-60% of mass 198
68	Less than 2% of mass 69
70	Less than 2% of mass 69
127	40-60% of mass 198
197	Less than 1% of mass 198
198	Base Peak, 100% Relative Abundance
199	5-9% of mass 198
275	10-30% of mass 198
365	1% of mass 198
441	Less than mass 443
442	40-60% of mass 198*
443	17-23% of mass 442

*This ion is very sensitive to spectrum number chosen and condition of

**ERL-N STANDARD OPERATING PROCEDURE
GAS CHROMATOGRAPHY-MASS SPECTOMETRY**

**ERL-N SOP 2.04.002
REVISION 0
FEBRUARY 1991
PAGE 5 OF 5**

equipment. If greater than 60% equipment is OK if all other criteria are met.

IV. TROUBLE SHOOTING

Discussed in methods section.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
GAS CHROMATOGRAPHY**

ERL-N SOP 2.04.003
REVISION 0
JANUARY 1991
PAGE 1 OF 4

POINT OF CONTACT:

Chemistry Group
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I. OBJECTIVES

The objective of this document is to define the standard procedure for analyzing marine environmental samples for polychlorinated biphenyls (PCBs) and chlorinated hydrocarbon pesticides using gas chromatography and electron capture detectors.

II. NECESSARY MATERIALS AND EQUIPMENT

- Hewlett Packard 5890 Gas Chromatographs equipped with electron capture detectors (Ni 63)
- automatic samplers
- 30 m DB-5 fused silica capillary columns (0.25 μ film thickness, 0.25 mm i.d.).
- Perkin-Elmer 3200 LIMS computer system with CLAS software -- provides for collection and storage of raw chromatographic data, and for selection and quantitation of analyte peaks.
- Ultra high purity helium, 95/5% Argon/Methane gases -- used as the carrier and auxiliary gas respectively.

III. OPERATION

A. Instrument checks made prior to data collection

1. Gas supply

- a. Check gas cylinder pressures. Replace tank if pressure is less than 100 psig.
- b. Check head pressure gauge on front panel of instrument. Gauge should read 18 psig; adjust to correct setting if reading is high; check for leaks if pressure is low. This setting provides for a carrier gas flow of approximately 1.5 ml/min.
- c. Replace injection port septum. Check septum nut and column fittings for leaks with leak detector and tighten as necessary.
- d. Check the auxiliary gas flow. A flow of 35 ml/min is required.
- e. Check septum purge and split flows. Adjust to 1 and 35 ml/min,

**ERL-N STANDARD OPERATING PROCEDURE
GAS CHROMATOGRAPHY**

ERL-N SOP 2.04.003
REVISION 0
JANUARY 1991
PAGE 2 OF 4

respectively, as necessary.

2. Instrument output signal

- a. Display the analog output signal from the detector on the LED panel of the GC.
- b. Record the value in the instrument log book, and check for consistency with previous readings. On instruments with dual detectors, ensure the signal is correctly assigned to the detector selected for the analysis.

3. Instrument operating parameters

- a. Temperature programs and run times are stored as workfiles in each GC's integrator. The following conditions are required for the analysis of PCBs and pesticides:

Injection port temperature	275°C
Detector temperature	325°C
Initial column temperature	100°C
Initial hold time	1 min
Rate 1	5°C/min
Ramp 1 final temperature	140°C
Ramp 1 hold time	1 min
Rate 2	1.5°C/min
Ramp 2 final temperature	230°C
Ramp 2 hold time	20 min
Rate 3	10°C/min
Final column temperature	300°C
Final hold time	5 min
Stop time	100 min
Injection port purge open time	1 min

- b. Load an appropriate workfile into the integrator.
- c. Enter the autosampler parameters into the integrator via Option 11. Indicate which injection port is being used, the number and positions of the samples in the autosampler tray, the number of injections per bottle, and the amount injected (1 ul).
- d. Check the signal assignments and levels again. If they are correct, store the workfile in the integrator.

B. Data system setup

1. Scheduling of standards and samples

- a. Setting up the instrument queue is accomplished by following instructions

laid out in the Perkin Elmer CLAS manual.

b. Order the samples, standards, and rinses according to the following guidelines:

- place hexane rinses before and after standards
- bracket groups of no more than five (5) samples with standards.
- arrange multiple level standards so that a high and a low standard precede as well as follow samples
- procedural and field blanks should be run prior to samples to minimize risk of carryover contamination.

c. Type in sample weight and internal standard amounts for each sample to be used in final concentration calculations. Double check all manually entered values for accuracy.

C. Instrument startup and data collection

1. After the instrument has been scheduled, arrange the samples and standards to be run in the autosampler trays. Check the order for accuracy against a copy of the queue. Load the trays into the autosampler.
2. Visually recheck tank regulator gauges and instrument settings to ensure proper settings.
3. Start GC operation and data collection by pressing 'start' on the integrator.

D. Peak identification and quantitation

1. Peak identification is accomplished by automated routines. Identifications are based on comparison of retention times of actual standards to unknown peaks. Multilevel standards are calibrated to generate a linear regression curve of response according to the manufacturer's instructions. After a calibration curve has been generated, the samples are analyzed. Analytes are quantitated based on the peak areas for the analytes and internal standard, the amount of the internal standard, and the response factors generated from the calibration curve. Chromatograms and data reports are generated for each sample and standard.

IV. QUALITY ASSURANCE AND TROUBLESHOOTING

1. Chromatograms of standards are compared to posted references. Peak identifications, resolution and shapes are inspected. Calculated standard amounts are checked for accuracy. Other abnormalities, such as spurious or extra peaks, rising or falling baselines, and negative spiking are examined. Response factors are compared to previous runs. Blanks are checked for the presence of interferences or analytes of interest. Unknown samples are compared to standards to check peak identifications.

**ERL-N STANDARD OPERATING PROCEDURE
GAS CHROMATOGRAPHY**

**ERL-N SOP 2.04.003
REVISION 0
JANUARY 1991
PAGE 4 OF 4**

2. Refer to the ERLN GC Troubleshooting notebook, the manufacturer's manuals, or to experienced personnel for guidance in troubleshooting the GCs.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not Applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
INORGANIC ANALYSIS BY FLAME ATOMIC ABSORPTION
SPECTROPHOTOMETRY**

**ERL-N SOP 2.04.004
REVISION 0
FEBRUARY 1991
PAGE 1 OF 3**

POINT OF CONTACT:

**Chemistry Group
US Environmental Protection Agency and
Science Applications International Corporation
c/o US Environmental Protection Agency
27 Tarzwell Dr.
Narragansett, RI 02882**

I. OBJECTIVE

This SOP describes the procedure for inorganic analysis by flame atomic absorption spectrophotometry.

II. NECESSARY MATERIALS AND EQUIPMENT

- Perkin-Elmer 5000 atomic absorption spectrophotometer.

III. METHOD

The manufacturer's recommended instrumental operating conditions are generally employed, except where ERL-N protocols are specified.

A. Instrumental Setup

1. Be sure that the instrument is in **STANDBY** mode and that the **POWER** is **ON**.
2. Toggle operating switch from **STANDBY** to **OPERATE**.
3. Turn on the strip chart recorder and set to **SERVO**.
4. If automated data collection is used, power up the data station, insert program and data disks into drives 0 and 1, respectively, and initiate **FLAME** data program.
5. Place the appropriate lamp in the turret and put power plug in the power socket for that turret position.
6. Enter the selected operating conditions from the keyboard.
 - a. Wavelength and slit width are selected from the instrument's standard methods handbook.
 - b. Lamp operating current/power is obtained from the label on the lamp or lamp box.

**ERL-N STANDARD OPERATING PROCEDURE
INORGANIC ANALYSIS BY FLAME ATOMIC ABSORPTION
SPECTROPHOTOMETRY**

**ERL-N SOP 2.04.004
REVISION 0
FEBRUARY 1991
PAGE 2 OF 3**

- c. If background correction is to be employed (generally necessary for wavelengths below 300 nm), set AA MODE to AA-BG.
 - d. Integration time should be set between 0.5 and 1.5 seconds, depending on the level of signal and baseline noise level, to obtain an acceptable level of variability in the measured absorbance (increased integration time yields reduced variability).
 - e. Set SIGNAL MODE to continuous (CONT).
 - f. Align the lamp in the turret by pressing SETUP on the keyboard, then adjusting the lamp position with the turret screws to maximize the energy readout; if the energy reaches 99, press GAIN to reset the detector amplification (gain).
7. Check that both air and fuel pressures are as specified in the instrument operating manual, then open the AIR valve first and then the fuel toggle valve. Check that flows are correct and press ignite switch. The flame should ignite within 10 seconds; if not, shut off fuel and air valves and determine if/where gas leaks are present.
8. After burner head has warmed up (approx. 5 minutes), aspirate a dilute standard containing the element of interest and adjust the burner head position controls (vertical, horizontal and rotational) to obtain the maximum net signal. Note that changes of burner head position can alter both the peak and the baseline absorbances; check baseline absorbance by aspirating deionized (DI) water.
9. Set SIGNAL MODE to HOLD and enter the number of reading per sample into AVG (generally, minimum of 3).
10. If automated data collection is to be used, press the PRINT button to turn on the light, indicating the communications line is available.

B. Calibration and sample analysis

- 1. Standards should be chosen to bracket range of expected concentrations and keep absorbances within range of 0.01-0.5 A. A minimum of 3 standards and a blank should be run.
- 2. Before initiating calibration, aspirate DI for 5 seconds and press AZ button to set baseline.
- 3. Place sample uptake tubing in the first standard solution and press READ; as many absorbance readings as the number entered for AVG will be taken and displayed on the instrument readout, followed by display of the mean absorbance for all the replicate readings. If data collection is in use, the individual readings will be transmitted to the program and stored on disk. Repeat for all the standards chosen.
- 4. Ascertain that the sensitivity observed for the element of interest is reasonably close (within 25 %) of the sensitivity specified for that wavelength in the standard conditions

**ERL-N STANDARD OPERATING PROCEDURE
INORGANIC ANALYSIS BY FLAME ATOMIC ABSORPTION
SPECTROPHOTOMETRY**

ERL-N SOP 2.04.004
REVISION 0
FEBRUARY 1991
PAGE 3 OF 3

handbook.

5. For analysis of samples, repeat the procedures of step (which step). Sample absorbances should be less than 0.7 A (absorbance units) and stay within the range of calibration standards. If sample concentrations exceed that of the highest standard, then a higher standard should be run; if absorbances exceed 0.7 A, solutions should be diluted and re-analyzed.

6. Periodically, e.g. after every ten samples, a standard should be re-analyzed to check for instrumental drift. Also, for every 15-20 samples analyzed, one sample, chosen randomly, should be spiked with a roughly equal concentration of the element of interest and re-analyzed. Spike recovery, defined as the difference in concentration between the spiked and unspiked samples divided by the concentration of the spike, should be within 90-110%; if not, matrix interferences with the analysis are indicated and must be investigated.

IV. TROUBLE SHOOTING

Discussed in methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Calibration curves relating absorbance to solution concentration are generated by linear or polynomial regression of absorbance against concentration for the calibration standards. If automated data collection was used, this is performed by the program CALIB.

2. The curves generated are then used to convert sample absorbances to concentrations. If CALIB is used, the sample ID, element, dilution and concentration data are stored in a file on diskette. The sample solution concentrations are combined with the prepared sample mass and volume data to calculate sample concentrations in terms of ug metal/g dry weight of sample.

3. Metals concentrations determined in standard reference materials (SRMs), prepared with the samples as unknowns, should be compared with reference values and confidence intervals to confirm accurate preparation and analysis of the metals.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
INORGANIC ANALYSIS BY ICP**

**ERL-N SOP 2.04.005
REVISION 0
FEBRUARY 1991
PAGE 1 OF 5**

POINT OF CONTACT:

**Chemistry Group
US Environmental Protection Agency and
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c/o US Environmental Protection Agency
27 Tarzwell Dr.
Narragansett, RI 02882**

I. OBJECTIVE

This procedure describes the analysis of metals by inductively coupled plasma (ICP) atomic emission spectrometry.

II. NECESSARY MATERIALS AND EQUIPMENT

- ARL Model 3410 ICP spectrometer

III. METHOD

A. Instrumental startup

1. Verify proper operating conditions:

- a. Torch gas supply valve, pressure = 75 psi
- b. Purge gas flow = 3 SCFH
- c. Spray chamber drain bucket < 75 % full
- d. Reservoir for autosampler rinse well full

2. Start sample pump

- a. Inspect tubing for flexibility, absence of cracks or excessive discoloration.
- b. Stretch tubing around pump head and close clamp.
- c. Place autosampler tip in deionized water and turn on pump.
- d. Verify steady, continuous uptake of water.

3. Ignite plasma

- a. Press "START Torch" button on front of ICP.
- b. Check gas flow controls inside torch box:

- 1. Coolant = 23 psi
- 2. Plasma = 22 psi
- 3. Carrier = 50 psi

c. Record time TORCH ON in instrument usage log & allow plasma to stabilize > 15 minutes (this is a good time to edit autosampler files, if needed).

1. If plasma ignites, but is extinguished when carrier gas flow initiates, allow gas flows to purge torch and repeat (which step); plasma should stay lit by 2nd or 3rd try.
2. If plasma fails to light or stay lit:
 - a. Verify application of RF power.
 - b. Clean & gap igniter and repeat.
 - c. If plasma still won't light, service call may be required.

4. Power up EPIC (ICP instrument control software) on computer.

- a. Turn on power to computer, printer and display, if needed.
- b. Turn up intensity and/or contrast on display as needed.
- c. Select "ICP Operations" from menu (F1).

B. Calibrate wavelength driver.

1. Select "Zero Calibration" from menu (F2).
2. Perform zero order calibration (F7).
3. While zero order calibration is performed, record RF generator parameters in instrument usage log.
4. Record reference wavelength position in instrument usage log.
5. Return to ICP operations menu (F10).

C. Normalize the appropriate task.

1. Select "Normalization" from menu (F7).
2. Select task, if necessary (F1).
3. Enter number of replicates (F3) (usually 3).
4. Select hard copy logging if printout desired (F6).
5. Perform normalization (F8).

a. If using autosampler:

1. Load wavelength calibration solution and normalization standards into autosampler.

**ERL-N STANDARD OPERATING PROCEDURE
INORGANIC ANALYSIS BY ICP**

ERL-N SOP 2.04.005
REVISION 0
FEBRUARY 1991
PAGE 3 OF 5

2. Advance autosampler to place first solution in position for autosampler.

3. Answer prompts:

- a. Run uninterrupted? Y
- b. Special Wavelength or Calibration? S
- c. Special Cal. sln queued up? Y

b. If not using autosampler:

1. Calibrate wavelengths.

- a. Special Wavelength or Calibration? S
- b. Place autosampler tip in wavelength calibration solution
- c. Special Cal. sln queued up? Y

2. Run normalization stds.

- a. Select normalization standard to run and enter standard number at prompt.
- b. Place autosampler tip in standard solution and respond "Y" to prompt.

c. Calculate new normalization coefficients.

- 1. After all normalization standards have been run, enter "-1" for standard number.
- 2. Current and new coefficients are displayed; coefficients deviating more than +10% from original calibration highlighted in red.
- 3. Continue (F10).

- a. If all or most of coefficients are within 10%, accept new coefficients "Y"
- b. If many coefficients deviate by more than 10% or are erratic, do not accept the new coefficients "N"; recalibration or repeat of normalization necessary

6. Return to ICP operations (F10)

D. Analyze samples.

- 1. Select task, if necessary (F2).
- 2. Select autosampler file to be used, if any (F3).
- 3. Enter the number of replicates (F4) (usually 3).
- 4. Select results file to store analytical results (F5).

- a. Select results file to append results to existing file (F2).
 - b. Enter file name to create new file (F5).
 - c. Return to analysis menu (F10).
5. Select hard copy logging to get printout during analysis.
6. Proceed to analysis options menu (F8) and select options as applicable, e.g. display intensities, dilution correction, etc.
7. Proceed to sample analysis menu (F8).
8. Begin analysis of sample(s).
 - a. If not using autosampler:
 1. Enter sample ID (F1) and weight and dilution corrections (F2 & F3), if desired.
 2. Place autosampler tip in sample solution and initiate analysis (F8).
 3. Repeat (**which step**) and (**which step**) for each sample.
 - b. If using autosampler:
 1. Advance autosampler to place first solution in position for autosampler
 2. Initiate analysis of samples (F8)
 3. After autosampler file has run through, more samples may be analyzed manually (I.D.8.a) or with another autosampler file
 - c. Return to analysis menu (F10, F10)
 1. To analyze more samples using an autosampler file, repeat I.D.2-8.
 2. If finished, turn off hardcopy logging if selected
 - d. Return to ICP operations (F10) and EPIC (F10)

E. Shutdown

1. Pump deionized H₂O through pump and spray chamber for > 15 minutes.
2. Stop pump, release clamp and remove tubing from pump head.
3. Press "STOP Torch" button and record time TORCH OFF in instrument usage log.
4. Turn display intensity all the way down.

IV. TROUBLE SHOOTING

Discussed in methods.

**ERL-N STANDARD OPERATING PROCEDURE
INORGANIC ANALYSIS BY ICP**

**ERL-N SOP 2.04.005
REVISION 0
FEBRUARY 1991
PAGE 5 OF 5**

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
INSTRUMENTAL OPERATING CONDITIONS FOR
INORGANIC ANALYSIS**

ERL-N SOP 2.04.006
REVISION 0
FEBRUARY 1991
PAGE 1 OF 2

POINT OF CONTACT:

Chemistry Group
US Environmental Protection Agency and
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I. OBJECTIVE

The conditions given below describe the particular instrumental parameters derived for atomic absorption and emission analysis of environmental samples at ERL-N.

II. NECESSARY MATERIALS AND EQUIPMENT

- ARL Model 3410 ICP spectrometer
- Perkin-Elmer 5000 atomic absorption spectrophotometer

III. METHOD

Where conditions for a particular element and instrument are not specifically provided, the instrument manufacturer's recommended operating conditions and parameters are used.

A. Inductively Coupled Plasma Operating Conditions

Element	Analytical Wavelength (nm)	Bkgd. Correction Wavelength(s) (nm)	Detection Limit (ug/ml)
Cu	324.754	324.793	0.010
Zn	213.856	213.820	0.005
Cr	205.552	205.588	0.010
Pb	220.353	220.311, 220.401	0.100
Ni	231.604	231.568	0.020
Cd	228.880	228.902	0.003
Mn	257.610	257.646	0.005
Fe	273.955	274.000	0.020 (sediments)
	259.940	259.985	0.010 (organism)

RF Power: 650 W forward, < 8 W reflected
Argon gas flows: Coolant 6.5 L/min
Plasma 1.0 "
Nebulizer 0.7 "

**ERL-N STANDARD OPERATING PROCEDURE
INSTRUMENTAL OPERATING CONDITIONS FOR
INORGANIC ANALYSIS**

ERL-N SOP 2.04.006
REVISION 0
FEBRUARY 1991
PAGE 2 OF 2

Sample solution pumping rate : 1.75 ml/min

B. Graphite Furnace Atomic Absorption Operating Conditions

Element	Wavelength (nm)	Slit (nm)	Ash temp deg C	Atom temp deg C	Matrix modifier
Ag	328.1	0.7	1000 900	1900 1900	100 ppm Pd/5 % H ₂ alt gas NH ₄ H ₂ PO ₄
As	193.7	0.7	1300	2100	250 ppm Pd/5 % H ₂ alt gas
Cd	228.8	0.7	1000	1800	100 ppm Pd/5 % H ₂ alt gas
Cu	324.7	0.7	1000	2300	
Fe	248.3	0.2	1400	2700	
Mn	279.5	0.2	1300	2200	
Ni	232.0	0.2	1400	2500	
Pb	283.3	0.7	1200	1900	250 ppm Pd/5 % H ₂ alt gas

Analyses are performed using L'Vov platforms, maximum power heating, zero-gas flow during atomization and Zeeman background correction. Peak areas used for calibration and quantitation. All analyses utilize 15 ul sample injections + 15 ul matrix modifier.

IV. TROUBLE SHOOTING

Discussed in methods section.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
DICHLOVOS MONITORING**

ERL-N SOP 2.05.001
REVISION 0
JANUARY 1991
PAGE 1 OF 2

POINT OF CONTACT:

Chemistry Group
US Environmental Protection Agency and
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Narragansett, RI 02882

I. OBJECTIVE

This method is used to monitor Dichlovos concentrations in water column samples.

II. NECESSARY MATERIALS AND EQUIPMENT

- Separatory funnels, 2 L
- Erlenmeyer flasks, 250 mL
- Methylene chloride (CH_2Cl_2)
- Internal standard IND9458906 (1,3 - Dimethyl-N-Benzene)
- Standard MIX0288906 (DCVOS @ 0.283ug/ul and 1,3-DMNB @ 0.164ug/ul in acetone)
- Na_2SO_4
- Boiling beads
- Round bottom flask
- Concentrator tube
- Hexane
- GC, Nitrogen Phosphorous detector (NPD)

III. METHOD

1. Rinse separatory funnels (2 liter) and an equivalent number of 250ml erlenmeyer flasks with Methylene Chloride (CH_2Cl_2).

2. Obtain water sample from monitoring system using syphon method. Amount of sample taken will be determined by expected concentration (ie. if sample conc. is 3,5, or 10ug/l, 1 liter of water is taken. If sample conc. is 50 or 150ug/l, 100ml of water is taken and for a conc. of 300ug/l, 50ml of water is taken).

3. Syphon sample into labeled sep. funnel and add 100ul of internal standard IND9458906 (1,3 - Dimethyl-N-Benzene @ 0.274 ug/ul). Rinse tubing with CH_2Cl_2 to ensure that the compound of interest is not adhering to the surfaces. Spike 500ml seawater with 200ul of standard MIX0288906 (DCVOS @ 0.283ug/ul and 1,3-DMNB @ 0.164ug/ul in acetone).

ERL-N STANDARD OPERATING PROCEDURE
DICHLOVOS MONITORING

ERL-N SOP 2.05.001
REVISION 0
JANUARY 1991
PAGE 2 OF 2

4. Extract 3 x 50ml CH₂Cl₂. Collect CH₂Cl₂ extract in pre-rinsed erlenmeyer flasks and dry with Na₂SO₄.
5. Rinse appropriate number of round bottom flasks with CH₂Cl₂ and add boiling beads.
6. Pour samples into round bottom flasks and reduce to ~10 ml HEXANE. (Solvent exchange during reduction)
7. Further reduce samples to 1.0ml hexane in pre-rinsed concentrator tubes.
8. Analyze samples on the GC using the Nitrogen Phosphorous detector (NPD), instrument#2.
9. Parameters for analysis are:

Workfile #4
Method DCVOS
Test DCVOS
Standard Wt. 27.4

Sample Queue:				SAMPLE	STAND	DILUT		
SEQ	SAMPLE	METHOD	TEST	DESC	WEIGHT	WEIGHT	FACTOR	C #R
001	KHEXANE	DCVOS		SOLV	1.0000	1.0000	1.0000	N 01
002	KDCVOS01	DCVOS		STD	1.0000	32.800	1.0000	Y 01
003	KHEXANE	DCVOS		SOLV	1.0000	1.0000	1.0000	N 01
004	0115...	DCVOS	DCVOS	WW	0.5000	27.400	1.0000	N 01

IV. TROUBLE SHOOTING

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

**ERL-N STANDARD OPERATING PROCEDURE
PROPOXUR MONITORING**

ERL-N SOP 2.05.002
REVISION 0
JANUARY 1991
PAGE 1 OF 3

POINT OF CONTACT:

Chemistry Group
US Environmental Protection Agency and
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I. OBJECTIVE

This method is used to monitor Propoxur concentrations in water column samples.

II. NECESSARY MATERIALS AND EQUIPMENT

- Separatory funnels, 1 or 2 L
- Erlenmeyer flasks, 250 mL
- Methylene Chloride (CH_2Cl_2).
- Carbaryl, 311ng/ul
- Sodium sulfate
- Turbo-Vap tubes.
- hexane
- Screw top 15 ml culture tube
- 0.18M ethanolic KOH (1.0 g KOH in 100ml 95% ethanol)
- Derivatizing agent (made by diluting 0.1 ml -bromo-2,3,4,5,6-pentafluorotoluene to 10 mls with 95% ethanol)
- Kontes tube heater
- Deionized water
- Graduated concentrator tube
- GC, Electron Capture Detector (ECD), and 30m DB-210 column

III. METHOD

1. Rinse separatory funnels (1 or 2 liter) and an equivalent number of 250ml erlenmeyer flasks with methylene chloride.
2. Obtain water sample from monitoring system using patented syphon method. Amount of sample taken will be determined by expected concentration (i.e. if sample conc. is 1-9ug/l, 1 liter of water is taken. If sample conc. is 10ug/l and above 500ml of water is taken.)
3. Syphon sample into labeled sep. funnel and rinse tubing with methylene chloride to assure that the compound of interest is not adhering to the sides. Add 100ul of internal standard (Carbaryl, 311ng/ul).

ERL-N STANDARD OPERATING PROCEDURE
PROPOXUR MONITORING

ERL-N SOP 2.05.002
REVISION 0
JANUARY 1991
PAGE 2 OF 3

4. Extract 3 x 50ml CH₂Cl₂. Collect CH₂Cl₂ extract in pre-rinsed erlenmeyer flasks. Add approximately 5 gms. sodium sulfate to dry. Transfer to pre-rinsed Turbo-Vap tubes.

5. Reduce sample volume to 0.5-1.0 ml using Turbo-Vap. As volume approaches 1 ml, add approximately 1 ml hexane to the sample tubes.

6. Derivatize the sample:

a. To a screw top 15 ml culture tube, add 1.0 ml of 0.18M ethanolic KOH (1.0 g KOH in 100ml 95% ethanol).

b. Transfer the sample to the culture tube.

c. Add 100 uls derivatizing agent (made by diluting 0.1 ml -bromo-2,3,4,5,6-pentafluorotoluene to 10 mls with 95% ethanol).

d. Place tubes in a Kontes tube heater set at 95 \pm 1° C for 2 hrs.

e. After 2 hrs., allow samples to cool. Add 5.0 mls D.I. H₂O and 4.0 mls. hexane. Vortex each tube for 10 sec.

f. Transfer the hexane layer to a graduated concentrator tube. Add 4.0 mls hexane and vortex. Combine hexane extracts and adjust volume to 10.0 mls, except in the case of lower concentrations where further volume reduction may be necessary.

7. Analyze samples on the GC using the Electron Capture Detector (ECD), and a 30m DB-210 column. Standards are made by treating an appropriate amount of Propoxur and carbaryl as sample in step six above.

8. Parameters for analysis are:

Workfile	#5	
Initial Temp.:	60	Inj. Temp.: 200
Initial Time:	1 min.	Det. Temp.: 230
Rate:	10/min.	
Final Temp.:	220	
Final Time:	13.5 min.	

Sample Queue:

SEQ	SAMPLE	METHOD	TEST	DESC	WEIGHT	STAND	WEIGHT	DILUT	FACTOR	C	#R
001	HEXANE	PPXUR		SOLV	1.0000		1.0000		1.0000	N	01
002	PPXOS01	PPXUR		STD	1.0000		31.100		1.0000	Y	01
003	HEXANE	PPXUR		SOLV	1.0000		1.0000		1.0000	N	01
004	0115...	PPXUR	PPXUR WW		0.5000		31.100		1.0000	N	01

**ERL-N STANDARD OPERATING PROCEDURE
PROPOXUR MONITORING**

**ERL-N SOP 2.05.002
REVISION 0
JANUARY 1991
PAGE 3 OF 3**

IV. TROUBLE SHOOTING

Not applicable.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

None.

**ERL-N STANDARD OPERATING PROCEDURE
CARBARYL MONITORING**

ERL-N SOP 2.05.003
REVISION 0
JANUARY 1991
PAGE 1 OF 3

POINT OF CONTACT:

Chemistry Group
US Environmental Protection Agency and
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27 Tarzwell Dr.
Narragansett, RI 02882

I. OBJECTIVE

This method is used to monitor water column samples for carbaryl.

II. NECESSARY MATERIALS AND EQUIPMENT

- Separatory funnels, 1 or 2 L capacity
- Erlenmeyer flasks, 250 mL
- Methylene chloride
- Propoxur
- Anhydrous sodium sulfate
- Turbo-Vap tubes
- Hexane
- Culture tube, 15 mL
- KOH
- Ethanol
- Bromo-2,3,4,5,6-pentafluorotoluene
- Kontes tube heater
- Deionized water
- Graduated concentrator tube
- GC, Electron Capture Detector (ECD), 30m DB-210 column

III. METHOD

1. Rinse separatory funnels (1 or 2 liter) and an equivalent number of 250 mL erlenmeyer flasks with Methylene Chloride (MeCl_2).
2. Obtain water sample from monitoring system using patented syphon method. Amount of sample taken will be determined by expected concentration (i.e. if sample conc. is 1-9 ug/L, 1 liter of water is taken. If sample conc. is 10 ug/L and above 500 mL of water is taken.)
3. Syphon sample into labeled sep. funnel and rinse tubing with methylene chloride to assure that the compound of interest is not adhering to the sides. Add 100 uL of internal standard (Propoxur, 311 ng/uL).

**ERL-N STANDARD OPERATING PROCEDURE
CARBARYL MONITORING**

**ERL-N SOP 2.05.003
REVISION 0
JANUARY 1991
PAGE 2 OF 3**

4. Extract 3 x 50ml MeCl₂. Collect MeCl₂ extract in pre-rinsed erlenmeyer flasks. Add approximately 5 g sodium sulfate to dry. Transfer to pre-rinsed Turbo-Vap tubes.

5. Reduce sample volume to 0.5-1.0 mL using Turbo-Vap. As volume approaches 1 mL, add approximately 1 mL hexane to the sample tubes.

6. Derivatize the sample:

a. To a screw top 15 mL culture tube, add 1.0 mL of 0.18M ethanolic KOH (1.0 g KOH in 100ml 95 % ethanol).

b. Transfer the sample to the culture tube.

c. Add 100 uL derivatizing agent (made by diluting 0.1 mL -bromo-2,3,4,5,6-pentafluorotoluene to 10 mL with 95 % ethanol).

d. Place tubes in a Kontes tube heater set at 95 +/- 1° C for 2 hrs.

e. After 2 hrs., allow samples to cool. Add 5.0 mL D.I. H₂O and 4.0 mL hexane. Vortex each tube for 10 sec.

f. Transfer the hexane layer to a graduated concentrator tube. Add 4.0 mL hexane and vortex. Combine hexane extracts and adjust volume to 10.0 mL, except in the case of lower concentrations where further volume reduction may be necessary.

7. Analyze samples on the GC using the Electron Capture Detector (ECD), and a 30m DB-210 column. Standards are made by treating an appropriate amount of Propoxur and carbaryl as sample in step six above.

8. Parameters for analysis are:

Workfile #5
Initial Temp.: 60
Initial Time: 1 min.
Rate: 10 /min.
Final Temp.: 220
Final Time: 13.5 min.

Inj. Temp.: 200
Det. Temp.: 230

VI. TROUBLE SHOOTING

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

**ERL-N STANDARD OPERATING PROCEDURE
CARBARYL MONITORING**

**ERL-N SOP 2.05.003
REVISION 0
JANUARY 1991
PAGE 3 OF 3**

VI. REFERENCES

None.

**UNH, JEL STANDARD OPERATING PROCEDURE
EELGRASS (*Zostera marina*) COLLECTION
AND POPULATION CHARACTERISTICS**

**JEL SOP 1.01
Revision 0
January 1992**

POINT OF CONTACT:

**Frederick T. Short
Jackson Estuarine Laboratory
University of New Hampshire
85 Adams Point Road
Durham, NH 03824**

I. OBJECTIVE

Biomass collection and assessment of eelgrass, including population characteristics and infection by the wasting disease organism *Labyrinthula zosterae*.

II. NECESSARY MATERIALS AND EQUIPMENT

- Protective clothing and gear for handling hazardous materials in the field (i.e., boots, gloves, safety glasses, apron, raingear).
- Small boat with standard safety equipment
- Eelgrass tongs: modified oyster tongs 25 cm wide with mud-holding slats on each side and set to open to 25 cm (1/16 m²)
- Plastic bags: 6 small (quart size) and 7 large (gallon size)
- Shellfish sorting basket (mesh opening 0.8 cm) with float (life ring)
- Sorting trays, biomass data sheets, metersticks
- Aluminum foil and marking pen
- Drying oven (80°C)
- Desiccator
- Balance

III. METHODS

A. Field collection

1. Select an area centrally located within the eelgrass bed after anchoring the vessel.
2. Lower the open tongs onto sediment with the rows of teeth parallel to the eelgrass blades. Push the teeth firmly into the mud. Close the tongs and extract the sample slowly; a rocking motion is often necessary to release the sediment suction.

UNH, JEL STANDARD OPERATING PROCEDURE
EELGRASS (*Zostera marina*) COLLECTION
AND POPULATION CHARACTERISTICS

JEL SOP 1.01
Revision 0
January 1992

3. At the surface, bring the tongs over the sieve basket, (often with help from another worker) and open as the other worker gently frees the mud and leaves from the tongs.
4. First, collect and place ten terminal, vegetative shoots with their longest leaf intact (no broken tip) in the smaller bag (roots rinsed of sediment). Wash remaining leaves, roots and rhizomes, including any macroalgae, free of sediment in water and place in the larger bag with the smaller bag.
5. Collect six biomass samples in this way and a seventh sample of live shoots, roots, and rhizomes that will be frozen and archived upon return to the lab.
6. Store the samples in a cooler while transporting to the lab. At the lab, rinse all samples with fresh water then keep the samples at 5°C in a dark container until they are processed.

B. Laboratory Processing

1. Work on one sample at a time, being sure to label all bags and containers carefully. Rinse the plants in fresh water and place them in an enamel tray containing 2 inches of tap water. Begin to fill out biomass and Wasting Index data sheets (attached), including your name.
2. For the 10 terminal, vegetative shoots (small bag) measure the width of a leaf, the length of the longest leaf and the number of leaves, and record this data on the biomass data sheet. If there are not 10 terminal shoots, only measure the terminal shoots that were collected. Place any lateral shoots in with the other vegetative shoot subsample. Remove epiphytes from all shoots by scraping each leaf, and discard scrapings. Separate older leaves on each shoot from very young, bright green leaves.
3. Using the Wasting Index data sheet, determine the extent of the wasting disease (*Labyrinthula zosterae* infection) on the first two terminal shoots as described on the Wasting Index Key. (The first two shoots from each of six samples will yield a sample size of 12 shoots for each station.)
4. For aboveground tissue (large bag), remove one shoot at a time from the pan by slowly pulling it out by its base while swishing the other shoots in the tray so as not to tangle and break any leaves. Remove epiphytes as above.
5. Count the number of shoots from the quadrat and record as density : # shoots/ area of quadrat, distinguishing between reproductive and vegetative shoots. On

the reproductive shoots, count and record the number of flower or seed bodies (spathes).

6. For below ground biomass (large and small bags), pick out live rhizomes and roots. Recognize them by their lighter color, lighter center when cut, turgid structure and tensile strength. Detritus (dead roots and rhizomes) will often be black and live material will often be light in color, but this is not always true. Break and separate rhizomes that are part live and part dead. Measure and record the total rhizome length. Sort dead roots and rhizomes (detritus) and living macroalgae from the sample.
- 7a. If samples are to be analyzed on a freshweight basis (for chemical analyses) pat dry and measure wet weight. Freeze immediately in ziplock plastic bags and label.
- 7b. If samples are to be analyzed dry place each subsample in a foil envelope (open at one end) labeled with the station code, date collected, and the sample location number (SL#), and what type of material is in the packet (10 shoots-Young leaves, 10 shoots-Old leaves, Vegetative shoots, Reproductive shoots, Roots & Rhizomes, Detritus, and Macroalgae). Place them all in a 80°C oven until dry (at least 24 hours). When dry, take the samples out of the oven and immediately fold over the top of each foil bag a few times to seal them and place in a desiccator. When cool, remove, measure and record each sample weight. Seal the foil envelopes up directly after weighing. Place them all in a ziplock bag labeled with project, station and date and place in correct storage box (for chemical analyses).

IV. TROUBLE SHOOTING / HINTS

Discussed in methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

Appropriate comparisons of mean values between stations and groups of stations can be made in an ANOVA framework, after ensuring that the assumptions of normality and homogeneity of variance are met.

VI. REFERENCES

- Short, F. T. 1987. The effects of sediment nutrients on seagrass growth and morphology. *Aquatic Botany* 27:41-57.

UNH, JEL STANDARD OPERATING PROCEDURE
EELGRASS (*Zostera marina*) COLLECTION
AND POPULATION CHARACTERISTICS

JEL SOP 1.01
Revision 0
January 1992

Short, F. T., D. M. Burdick and J. Wolf. A wasting index to assess and monitor the progression of the wasting disease, *Labyrinthula zosterae*, in eelgrass, *Zostera marina*. in prep.

UNH, JEL STANDARD OPERATING PROCEDURE
EELGRASS (*Zostera marina*) COLLECTION
AND POPULATION CHARACTERISTICS

JEL SOP 1.01
Revision 0
January 1992

SEAGRASS BIOMASS				LAND SIZE		BIOMASS FORM	
Date collected				above gr.			
Date analyzed				below gr.			
Location							
Site #	Shoot #	Length (cm)	Width (mm)	# of shoot	DENSITY		DRY WT. (gr)
Core #	1				SHOOT DC	10 shoots old	
	2					10 shoots YG	
	3				# Shoots	total shoots	
	4					flowers	
	5				rhiz. leng (cm)	rhiz. & rhiz	
	6					detritus	
	7					macroalgae	
	8						
	9						
	10						

Comments

UNH, JEL STANDARD OPERATING PROCEDURE
EELGRASS (*Zostera marina*) COLLECTION
AND POPULATION CHARACTERISTICS

JEL SOP 1.01
Revision 0
January 1992

EELGRASS WASTING INDEX DATA ANALYSIS																
Date:	Width	Sheath	Leaf #1	Index	Leaf #2	Index	Person:	Leaf #3	Index	Leaf #4	Index	Leaf #5	Index	Leaf #6	Index	Comments
Shoot #	(mm)	(cm)	Length	%	Length	%	Length	%	Length	%	Length	%	Length	%		
1																
2																
3																
4																
5																
6																
7																
8																
9																
10																

UNH, JEL STANDARD OPERATING PROCEDURE
EELGRASS (*Zostera marina*) COLLECTION
AND POPULATION CHARACTERISTICS

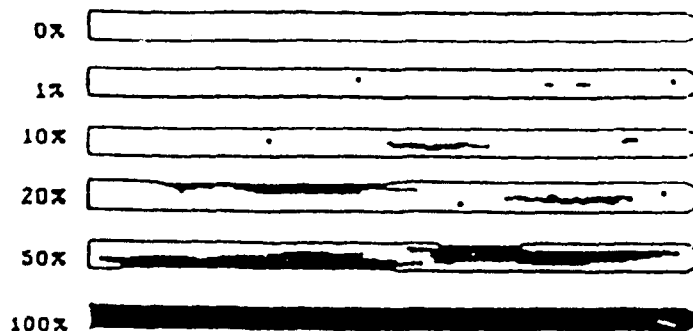
JEL SOP 1.01
Revision 0
January 1992

WASTING INDEX METHOD

Introduction: The purpose of the wasting index method is to have an easy and quantitative way to determine the amount of disease on an eelgrass shoot.

- A. Enter the date the plants were collected under "Date," the location and site the plants were collected at under "Site," and the person collecting the eelgrass and measuring the disease under "Person."
- B. Select a terminal, vegetative shoot and number it. Enter the number on the data sheet under "Shoot #."
- C. Measure the shoot width in millimeters (e.g. 3.2) and enter under "Width."
- D. Measure the height of the youngest visible sheath (usually encloses the youngest two to three leaves) from the youngest root node in centimeters (e.g. 14.7) and enter under "Sheath."
- E. Number the leaves of each shoot from youngest to oldest.
- F. Measure length of each leaf from the youngest root node to the tip in centimeters (e.g. 54.9) and enter under "Length." If the tip is broken, measure to break and write "BT" next to measurement.
- G. Enter percentage of disease on the leaf under "Index." To estimate the percentage of disease on a leaf look at the entire leaf from the top of the sheath to the tip, then refer to the "Wasting Index Key." On this key is drawn the amount of disease for 0, 10, 20, 50, and 100 percent. Estimate where the leaf you are looking at stands on this sheet. You should interpolate if it appears to have a percentage of disease between the numbers on the key (e.g. 3% or 85%).
- H. Enter anything that seems abnormal or worthy noting under "Comments."

INDEX KEY



UNH, JEL STANDARD OPERATING PROCEDURE
EELGRASS (*Zostera marina*) CARBON, NITROGEN,
AND PHOSPHORUS

JEL SOP 1.02
Revision 0
January 1992

POINT OF CONTACT:

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Jaimie Wolf
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I. OBJECTIVE

This SOP describes procedures for determining the concentrations of carbon, nitrogen, and phosphorus (CNP) in eelgrass leaves. See JEL SOP 1.01 for collection and preparation of eelgrass leaves.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Equipment

- Protective clothing and eyewear for handling hazardous materials
- Data sheets, marking pen
- Wiley Mill, 40 mesh screen
- Combustion boats
- Balance ± 0.001 g
- Balance ± 0.001 mg
- Carlo-Erba elemental analyzer, Model 1500 (CNS analysis)
- Dry, HCl washed test tubes (12×125 mm), with marbles or glass stoppers
- Dry, HCl washed 125 ml Erlenmeyer flasks
- 1 L beakers
- 0.5 ml pipette
- 50 ml pipette
- 200 ml volumetric flask
- 0.45 μ M filters, filter holders, gaskets
- 8 ml pipette
- 20 cc syringe
- 4 or 10 cm cells for spectrophotometer
- Perkin-Elmer Coleman 55 Spectrophotometer

B. Reagents

Oxidizing Agent - Add 5.0 g $K_2S_2O_8$ to 95 ml distilled water. Make enough for 8 ml per sample.

UNH, JEL STANDARD OPERATING PROCEDURE
EELGRASS (*Zostera marina*) CARBON, NITROGEN,
AND PHOSPHORUS

JEL SOP 1.02
Revision 0
January 1992

Ammonium Molybdate Solution - Dissolve 15.0 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in 500 ml distilled water. Store in a dark bottle without sun. This is stable indefinitely. Use as long as solution is clear. Refrigerate.

Sulfuric Acid Solution - Add 140 ml concentrated H_2SO_4 to 900 ml distilled water (or 62 ml acid to 400 ml water). Store in a glass bottle. Always add acid to water. Use the hood with window down, fan on, and wear gloves.

Ascorbic Acid Solution - Dissolve 5.4 g ascorbic acid in 100 ml distilled water - store in small plastic bottles in 20 ml amounts, frozen.

Potassium Antimonyl-Tartrate Solution - Dissolve 0.34 g $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$ in 250 ml distilled water - this may be warmed to help dissolve. The solution is stable for months and should be stored in a glass bottle. Use as long as solution is clear.

Mixed Reagent - Use 10 ml ammonium molybdate solution + 25 ml sulfuric acid solution + 10 ml ascorbic acid solution + 5 ml antimony solution or some multiple of the above (these volumes are sufficient for 10 samples). Prepare for immediate use; don't store for more than 6 hours or the solution will yellow due to ascorbic acid. Use the same graduated cylinder to eliminate error. If reagent turns at all blue or bluish green, discard.

C. Standards

Phosphate Solutions -

6000 μM P: Add 0.816 g anhydrous KH_2PO_4 to distilled water to make 1 L. Store in a dark plastic bottle with 1 ml chloroform in the refrigerator - stable for many months. If there is any doubt that the KH_2PO_4 is dry, place in oven for at least 10 hours at 110°C .

60 μM P: Dilute 10 ml 6000 μM KH_2PO_4 in distilled water to make 1 L. Store in a dark plastic bottle with 1 ml chloroform in the refrigerator. Replace every 10 days.

3.0 μM P: Dilute 10.0 ml 60 μM KH_2PO_4 to 200 ml (in a volumetric flask). This may be stored overnight only if refrigerated with 1 ml chloroform.

III. METHODS

A. General:

1. The subsample to be used in these analyses is the "10 Shoots - Young leaves" portion from the collection and biomass procedure (JEL SOP 1.01).
2. Grind the dried sample in a Wiley Mill using the 40 mesh screen and store the powder in a stopper vial (70 mg needed).

B. Carbon and Nitrogen Analysis on an Elemental Analyzer:

1. Follow procedures for preparing and weighing standards and samples, column packing, combustion, and GC analysis for the Carlo Erba Model 1500 Nitrogen Analyzer.
2. Be sure to purchase certified standards from Carlo Erba or authorized distributors.
3. Run standards as unknowns (samples) every 10 samples.
4. Record data for % of C and N for standards and samples synthesized by the DP 110.

C. Total Phosphorus, Spectrophotometric Determination:

1. Weigh out about 30 mg dry powdered plant material and place in test tube. Record weight.
2. Add 8 ml of 5% $K_2S_2O_8$ solution and cover the test tube with a marble or stopper (loosely).
3. Place test tubes in a 1 L beaker filled about halfway with water and a few boiling chips.
4. Warm water to boiling slowly over a period of 30 min. then boil water for 1 to 1.25 hr.
5. After cooling, check to be sure the total volume is still 8 ml (add distilled water if needed), then filter each solution into a polyethylene bottle for freezing until analysis.

UNH, JEL STANDARD OPERATING PROCEDURE
EELGRASS (*Zostera marina*) CARBON, NITROGEN,
AND PHOSPHORUS

JEL SOP 1.02
Revision 0
January 1992

6. Dilute 0.5 ml sample with distilled water to 50 ml in an Erlenmeyer flask.
7. Add 5 ml of mixed reagent, mix well.
8. After 5 min, measure absorbance (A_s) at 660 nm.
9. Calibration: Reagent blank, A_{rb} - steps 3, 4 on 50 ml of distilled water - do twice and average - this should not exceed 0.020.
10. To determine F factor: prepare four standards using 3.0 μM KH_2PO_4 using 50 mls of standard and steps 3, 4, find the mean of the four replicate A_s absorbances.

$$A_f = A_s - A_{rb} \quad F = 3.0/A_f$$

11. Concentration (C) in μM : $C = (A_s - A_{rb}) \times F$ If sample has been diluted, multiply concentration \times dilution.
12. Atom Percentage P: $\%P = C(\mu\text{M}) \times (1 \text{ L}/1,000 \text{ ml}) \times 8 \text{ ml} \times (31 \text{ g/mole}) \times (1 \text{ g}/106 \text{ g}) \times (1/\text{g sample}) \times 100.$

$$\text{or: } \%P = [C(\mu\text{M})/\text{wt}(\text{mg})] \times 2.478 \times 10^{-2}$$

Notes:

1. Let the spectrophotometer warm up for an hour before use.
2. Absorbance limitation for cells - 0.005 for 10 cm cell, $0.125 \pm .001$ for 4 cm cell.
3. The spectrophotometer is zeroed using water as the reference.
4. Range of detection is 0.01 to 5% P.
5. Samples should be shaken before being dispensed into flasks, and before being placed into cell for absorption reading.

IV. TROUBLE SHOOTING / HINTS

See methods and notes.

V. STATISTICAL ANALYSIS AND DATA USAGE

For carbon and nitrogen, the elemental analyzer calculates sample concentrations ($\pm 0.01\%$) based on standards and sample weights that are entered into the analyzers computer by the user. The mean of the two replicates is calculated for each sample. For phosphorus, the sample concentrations are calculated in Methods section C (10 & 11). Calculate means of duplicate replicates and report as a percentage. Atomic ratios of carbon, nitrogen and phosphorus should be calculated using P as the standard (i.e., XC:YN:1P).

VI. REFERENCES

- Carlo Erba Strumentazione Nitrogen Analyzer Model 1500 Instruction Manual.
P.O. Box 10364-1 20110 Milan - Cable Erbadass - Milan, Italy.
- Menzel, D. W. and N. Corwin. 1965. The measurement of total phosphorus in seawater based on the liberation of organically bound fraction by persulfate oxidation. *Limnology and Oceanography* 10:280-282.
- Short, F. T. 1990. Primary elemental constituents. pp. 105-109. In: Seagrass Research Methods. R. C. Phillips and C. P. McRoy, eds., Volume 9, Monographs on Oceanographic Methodology, UNESCO, France.
- Strickland, J. D. H. and T. R. Parsons. 1968 A Practical Handbook for Seawater Analysis. Fisheries Research Board of Canada, Ottawa. pp 52-55.

**UNH, JEL STANDARD OPERATING PROCEDURE
SEAWEED COLLECTION AND POPULATION
CHARACTERISTICS**

JEL SOP 1.03
Revision 0
January 1992

POINT OF CONTACT:

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I. OBJECTIVE

Determine the abundance and species composition of attached intertidal seaweeds and collect samples for chemical tissue analysis.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Field Collection

- Appropriate clothing for suspected contaminated conditions:
 - boots
 - raingear
 - gloves
 - glasses
- 1/16 m² quadrat
- putty knives
- bags (6 per site)
- single edge razor blades

B. Lab Processing

- Appropriate clothing for suspected contaminated conditions:
 - gloves
 - rubber apron
 - safety glasses
- Rinse trays
- Aluminum foil for drying trays
- Balance

III. METHODS

1. Wear protective clothing for collecting and handling benthic rocky intertidal samples.

**UNH, JEL STANDARD OPERATING PROCEDURE
SEAWEED COLLECTION AND POPULATION
CHARACTERISTICS**

**JEL SOP 1.03
Revision 0
January 1992**

2. Take random stratified samples in duplicate at three elevations: top, middle and lower levels of the fucoid belt. Design sampling program to ensure that all fucoid types are sampled.
3. Scrape all attached algae within a 1/16 m² quadrat frame with a putty knife and razor blade and put into individually labeled bags. Harvest the entire benthic communities in each sample/site, including mussel, periwinkle and seaweeds (mostly fucoids).
4. Upon return to the laboratory keep samples cool (5°C) until processing (within 7 days).
5. Sort each sample to species and record the damp-dried wet weight of each species enumerated. Enumerate the total number of each invertebrate species.
6. Set aside a 250 g (wet-weight) sample of the fucoid samples for air drying and future tissue analyses. Record the dry weights after reaching a constant weight. Following final sorting, return excess plant and animal samples to their initial collecting sites.

IV. TROUBLE SHOOTING

Define the extent of the fucoid belt at low tide and sample lower level replicates first to extend sampling time.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Make a comparison of wet/dry weight ratios of each fucoid sample to estimate plant standing crop/m².
2. Analyze species composition.

VI. REFERENCES

- Hardwick-Witman, M. A. and A. C. Mathieson. 1983. Intertidal macro-algae and macro-invertebrates: seasonal and spatial abundance patterns along an estuarine gradient. *Estuarine Coastal and Shelf Science* 16:113-129.
- McIntyre, A. D. 1984. *Methods for the Study of Marine Benthos*, 2nd ed. Blackwell Scientific, Oxford, pp. 387.

**UNH, JEL STANDARD OPERATING PROCEDURE
BLUE MUSSEL (*Mytilus edulis*) COLLECTION
AND POPULATION CHARACTERISTICS**

JEL SOP 1.04
Revision 0
January 1992

POINT OF CONTACT:

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I. OBJECTIVE

Determine the abundance and size distribution of the blue mussel and collect samples for chemical tissue analysis.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Field Collections

- Appropriate clothing for suspected contaminated conditions (boots, gloves, safety glasses, raingear)
- Waders for intertidal and shallow (<1 m) subtidal collections
- Modified oyster tongs for subtidal collections (modified so sample collected equals approximately 0.06 m²)
- Boat with standard marine safety equipment
- Distilled water for emergency eye wash
- 20 m floating line with attached end weights
- 10 mesh collection bags
- 5 gallon bucket with bottom cut off (0.06 m²)
- Garden claw trowel
- 10 plastic bags (1 gallon size) with suitable closures
- Sample identification tape
- Permanent markers
- Waterproof notebook and pencils
- Cooler with ice

B. Lab processing

- Appropriate clothing for handling of contaminated samples (apron, gloves, safety glasses)
- Set of graduated beakers (100 ml, 500 ml, 2000 ml)
- Rinse trays (The mesh in the rinse trays measures 0.55 cm by 0.7 cm. Therefore mussels excluded from sample are those measuring from 0 to 1.1 cm, and those included are those >1.1 cm.)
- Calipers
- Shucking knife

**UNH, JEL STANDARD OPERATING PROCEDURE
BLUE MUSSEL (*Mytilus edulis*) COLLECTION
AND POPULATION CHARACTERISTICS**

**JEL SOP 1.04
Revision 0
January 1992**

- Forceps
- Balance scale
- Weighing trays
- Drying oven (80°C)
- Shipping materials

III. METHODS

At each station *M. edulis* are collected by modified oyster tongs in subtidal areas, and walking transects on shore in intertidal areas using bucket samplers.

A. Field Collection

1. Define two 20 m transect lines at each station that are near the center of the bed, perpendicular to the shore, and 10 m apart from each other. Along the transect line, starting at 0 m, collect mussels every 5 m for a total of 5 samples per transect line.
- 2a. In subtidal areas collect mussels from the deck of a small boat using the modified oyster tongs along the floating transect line
- 2b. In the intertidal area place a bucket sampler (with the bottom cut off) to the right of the transect line, at the center of the 5 m mark on the line. Press the bucket down into the substrata and collect the resulting "core" of mussels (dead and alive) and place in one of the small mesh bags. If the substrate is rock, use the bucket as a guide and include the mussels that fall more than halfway inside the perimeter of the bucket.
3. Put samples into separate labeled plastic bags and place in a cooler for transport to the lab.
4. Collect extra mussels from the station if it appears that there will not be a total of 45 mussels >5 cm in length after 10 are removed from each of the 10 subsamples.

B. Laboratory Processing

1. Sort each bag of mussels, separating live mussels from dead and record the number of each (when counting dead mussels only record pairs of shells). Measure and record the volume of the remaining live mussels.

**UNH, JEL STANDARD OPERATING PROCEDURE
BLUE MUSSEL (*Mytilus edulis*) COLLECTION
AND POPULATION CHARACTERISTICS**

**JEL SOP 1.04
Revision 0.
January 1992**

2. From each bag take 10 mussels at random and place in a plastic bag marked with the Station # and its preassigned ID number and seal the bag. For each station there will be 10 bags of 10 mussels each, totaling 100 mussels.
3. Measure and record the shell length of these 100 mussels.
4. From each of the 10 bags take three mussels at random and record the volume. Discard the rest. For each one of the 30 mussels measure the shell length, remove and weigh the meat, dry at 80°C for 24 hours (or until at constant weight), weigh, and store for chemical tissue analysis.
5. From the mussels not included in the random sample choose 45 that are greater than 5 cm in length. Place and seal these in a plastic freezer bag marked with the Station # and its preassigned ID number. Place the bags in the freezer for later chemical analysis.
6. Return the remaining mussels and shells to the site.

IV. TROUBLE SHOOTING / HINTS

Discussed in methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Calculate relationship between length, volume, meat wet/dry weights
2. Determine means of meat weights (dry/m²), # mussels (live and dead), wet volume and above relationships.
3. Graph population size frequency

VI. REFERENCES

Newell, Carter. Great Eastern Mussel Farm. P.O. Box 141, Tenants Harbor, ME 04860.

**UNH, JEL STANDARD OPERATING PROCEDURE
WATER SAMPLING FOR SUSPENDED SOLIDS,
CHLOROPHYLL, pH AND NUTRIENTS**

**JEL SOP 1.05
Revision 0
January 1992**

POINT OF CONTACT:

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I. OBJECTIVE

This SOP explains the field procedure for obtaining water column measurements of the physical parameters of temperature, salinity, dissolved oxygen and for the collection of water samples for pH measurement and analysis of suspended solids, chlorophyll *a*, phaeopigments, ammonium, phosphate and nitrate-nitrite.

II. MATERIALS AND EQUIPMENT

A. Field Materials

- 1 Liter acid washed polyethylene bottles rinsed 5 times with distilled water (2 replicates/station recommended)
- Labels on bottles with station, replicate number, and date
- Coolers with ice for transport
- Protective rubber gloves
- Foul weather gear

B. Field Equipment:

- A boat of suitable size for wind and sea conditions and sample site locations
- YSI Model 33 conductivity, salinity, and temperature meter (or equivalent) with cable marked at 1 meter intervals
- YSI Model 58 dissolved oxygen meter (or equivalent) with cable marked at 1 meter intervals.
- Fisher Acumet Model 955 field pH meter or equivalent.

III. METHODS

A. Instruments:

1. Calibrate the YSI Model 33 CST meter and check batteries before each day's use. Store the probe in distilled water at all times when not in use..
2. Calibrate the YSI Model 58 D.O. meter and check batteries, membrane, and fluid, replacing when needed.

**UNH, JEL STANDARD OPERATING PROCEDURE
WATER SAMPLING FOR SUSPENDED SOLIDS,
CHLOROPHYLL, pH AND NUTRIENTS**

**JEL SOP 1.05
Revision 0
January 1992**

3. Calibrate the pH meter and rinse with distilled water after each use.
4. Soak all 1 liter bottles and caps for 24 hours in a 10% HCl solution, then drain and rinse 5 times with distilled water.

B. Sampling

1. Anchor the boat at the sampling site.
2. Turn on CST and D.O. meters and lower probes to the 1 meter mark. Allow at least 30 seconds at each depth for d.o. probe to equilibrate. Be sure conductivity and oxygen probes are more than 6" apart. Record readings and lower to next meter mark until the entire water column is measured. Probes should not touch substrate. In areas of strong tidal currents, small weights (1-2 lbs) attached to the cables at least 6" from the probes are recommended.
3. Water samples are subsurface grabs. Uncap, fill, and recap bottles while holding the bottles below the surface. Take care to avoid sampling the surface film. Measure and record pH immediately. Place the bottles in a cooler containing ice, keep in the dark, and filter within one hour of collection.

IV. TROUBLESHOOTING / HINTS

Discussed in Methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

Replicates are averaged, and these means are used in further analyses. Appropriate comparisons of mean values between stations and groups of stations can be made in an ANOVA framework, after ensuring that the assumptions of normality and homogeneity of variance are met.

VI. REFERENCES

None available.

**UNH, JEL STANDARD OPERATING PROCEDURE
WATER SAMPLE FILTRATION AND ANALYSIS OF
TOTAL SUSPENDED SOLIDS, CHLOROPHYLL AND
PHAEOPIGMENTS**

JEL SOP 1.06
Revision 0
January 1992

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I. OBJECTIVE

This SOP describes the procedure for processing seawater or estuarine water samples for analysis of suspended solids, chlorophyll *a*, phaeopigments and dissolved nutrients. It also describes the analytical methods for both suspended solids and chlorophyll/phaeopigments.

II. MATERIALS AND EQUIPMENT

- Vacuum pump
- Pre-dried and pre-weighed glass fiber filters (1.2 μ m nominal pore retention)
- Cahn Model 9500 digital microbalance
- Acid washed filter funnel and flask
- Deionized water
- 2% Magnesium carbonate suspension in water
- Filter forceps
- Labeled 50 ml centrifuge tubes
- Labeled 15 ml polypropylene centrifuge tubes
- Labeled 125 ml, acid cleaned polyethylene bottles (3 per water sample)
- Freezer
- Drying oven set at 80°
- Aluminum weighing dishes
- Muffel oven set at 500°C
- Motorized Thomas 20 ml tissue grinder with attached serrated teflon pestle
- Exhaust hood
- 90% acetone
- Pointed probe
- Test tube mixer (vortex type)
- Centrifuge, table top (3000 rpm max)
- Beckman Model 35 Spectrophotometer with 4 cm cuvettes
- Non scratching wipes
- Lens paper
- Automatic pipettor
- 50% Hydrochloric acid

**UNH, JEL STANDARD OPERATING PROCEDURE
WATER SAMPLE FILTRATION AND ANALYSIS OF
TOTAL SUSPENDED SOLIDS, CHLOROPHYLL AND
PHAEOPIGMENTS**

JEL SOP 1.06
Revision 0
January 1992

III. METHODS

A. Filtration

1. Attach filtration flask to a vacuum pump. Place a pre-dried, pre-weighed (fresh weight = w1) filter on the funnel base. Filter 500 ml of the sample through the funnel. Be sure to invert the water sample bottle several times to resuspend solids that may have settled. Remove the funnel apparatus from the flask and divide filtrate evenly into three acid cleaned 125 ml polyethylene bottles. These bottles should be labeled with station number, date, and type of analysis: ammonium, phosphate, or nitrate. These samples should be analyzed immediately, or frozen at -80°C for no more than one week before analysis. Replace funnel apparatus with filter still on base on the funnel. Using a squeeze bottle, rinse the filter to remove salts with approximately 2 ml of deionized water and filter the water through the funnel. Remove the filter with forceps, place in a labeled aluminum weighing dish and then into a drying oven set at 80°C for 24 hours, for determination of total suspended solids. The number on the aluminum dish should be etched since most inks are removed during later combustion.
2. Place a glass fiber filter of the same pore size on the funnel base. Invert same sample bottle several times and filter 500 ml of sample through the filter. When there is approximately 25 ml of water remaining in the funnel, add 1 ml of magnesium carbonate suspension. Using forceps, carefully fold the filter into quarters and place into a clean, dry 50 ml centrifuge tube. Keep the tube in the dark and refrigerated for up to one week for chlorophyll analysis.
3. Between samples, rinse and drain the filter flask and funnel with deionized water.

B. Suspended solids and % organic analysis

1. Remove dried suspended solids filters from the drying oven, place in a desiccator to cool for at least 0.5 hrs. Using the same balance used to weigh the clean filters, weigh the filters to the nearest tenth of a milligram (w2). Subtract the weight of the clean filters and divide by the volume of water filtered (in this case, 0.5 l) to obtain the suspended solids expressed in mg/l.

$$\text{TSS (mg/l)} = (w2 - w1) / 0.5 \text{ (liters)}$$

**UNH, JEL STANDARD OPERATING PROCEDURE
WATER SAMPLE FILTRATION AND ANALYSIS OF
TOTAL SUSPENDED SOLIDS, CHLOROPHYLL AND
PHAEOPIGMENTS**

JEL SOP 1.06
Revision 0
January 1992

2. Return the filter to the labeled aluminum weighing dish and combust it in a muffle oven (pre-heated to 500°C) for 24 hrs. Carefully remove the filters and allow to cool in a desiccator for at least one hour before weighing. Weigh the filters (w3) on the same balance. Determine % organic content by the following formulae:

$$w_{tss} = w2 - w1; \quad w_{ash} = w3 - w1$$
$$\% \text{ organic content} = [(w_{tss} - w_{ash}) / w_{tss}] \times 100$$

C. Chlorophyll *a* and phaeopigments

1. Extraction

Homogenization:

- a) Prepare 90% acetone using pure acetone and deionized water.
- b) Attach a serrated teflon pestle of a Thomas 20 ml tissue grinder to the drive assembly of the motorized grinder under an exhaust hood.
- c) Using forceps, place the sample filter into a grinding vessel.
- d) Using a squeeze bottle containing 90% acetone, squirt a few ml into the grinding vessel.
- e) Turn on the grinder and carefully insert the teflon pestle into the grinding vessel. Gently force the pestle up and down for several minutes to macerate the filter and disrupt algal cells. Be careful that no large pieces of the filter remain, yet no heat is generated by the grinding friction.
- f) Slowly lower the grinding vessel below the pestle while the grinder is still running. As the vessel is lowered, rinse the pestle with 90% acetone into the vessel. This should only require a few ml of acetone.
- g) Turn off the grinder. With a pointed probe, gently remove any glass fibers remaining on the pestle and add them to the liquid in the grinding vessel. Rinse the pestle with 90% acetone into the vessel. This should only use 3-5 ml of acetone.
- h) Stir the grinding vessel on the test tube mixer and quickly pour the suspension into a labelled 15 ml polypropylene centrifuge tube. Using the squirt bottle with 90% acetone, rinse any material remaining in the vessel into the centrifuge tube. Bring the volume of the centrifuge tube to exactly 15 ml with 90% acetone.
- i) Cap the tube and invert several times to mix completely. Immediately place tube in a refrigerator in the dark. Allow extraction to proceed overnight. To mix, invert samples 2 or 3 times during extraction.

**UNH, JEL STANDARD OPERATING PROCEDURE
WATER SAMPLE FILTRATION AND ANALYSIS OF
TOTAL SUSPENDED SOLIDS, CHLOROPHYLL AND
PHAEOPIGMENTS**

**JEL SOP 1.06
Revision 0
January 1992**

Centrifugation:

- a) Mix sample in centrifuge tube and place in centrifuge buckets. Be sure samples are paired in centrifuge. Centrifuge for 5 minutes at full speed.
- b) Gently pour off centrifugate into a new labeled 15 ml centrifuge tube. Do not disturb the fiber pellet at the bottom. It is not critical to transfer the entire volume of acetone extract - just most of it.
- c) Recentrifuge the acetone extracts as described in steps a and b.
- d) Pour all centrifuged samples into clean, labelled 15 ml centrifuge tubes.

2. Spectrophotometry

- a) Turn on spectrophotometer (power and absorbance buttons). Be sure that microcell aperture switch is on standard (not micro) and mirror level is on visible (not UV). Allow spec to warm up for at least 30 minutes before taking readings.
- b) Rinse the two 4 cm cuvettes with 90 % acetone, fill to near top and cap. Place both cuvettes in cuvette holder. Be sure that the cuvettes are set in tightly and level.
- c) Set wavelength to 750 nm. Always approach final setting from a higher wavelength. Zero the absorbance with baseline adjust knob to read "0.000".
- d) Remove front (sample) cuvette and empty. Rinse cuvette with a few drops of sample and discard. Fill cuvette to near top with sample and cap. Blot dry any acetone on cuvette with wipes and clean optical surface with lens paper.
- e) Replace cuvette into cuvette holder. Read absorbance at 750 nm (it will take several seconds to stabilize).
- f) Adjust wavelength to 665, 645, and 630 nm and record each absorbance. Return to 750 nm. Remove sample cuvette, pour sample back into centrifuge tube and cap tube.
- g) Repeat steps d, e, f for each sample.
- h) For the first few samples the spectrophotometer should be rezeroed. Rinse the cuvette several times with 90% acetone, then fill and repeat steps a, b, c. The spectrophotometer should then be rezeroed every 6-8 samples.
- i) After all samples have been read, using an autopipettor add 0.25 ml of 50% HCl to each centrifuge tube. Recap tubes and invert several times. Wait 15 minutes and repeat steps d-f.

D. Calculations

- a) Subtract each 750 nm reading from corresponding absorbances at 665, 645, 630.

**UNH, JEL STANDARD OPERATING PROCEDURE
WATER SAMPLE FILTRATION AND ANALYSIS OF
TOTAL SUSPENDED SOLIDS, CHLOROPHYLL AND
PHAEOPIGMENTS**

JEL SOP 1.06
Revision 0
January 1992

b) Calculate chlorophyll and phaeopigment concentrations with the formulas:¹

$$\text{Chl } a \text{ (mg/m}^3\text{)} = \frac{26.7 (665o - 665a) \times v}{V \times l}$$

$$\text{Phaeopigments (mg/m}^3\text{)} = \frac{26.7 (1.7 [665a] - 665o) \times v}{V \times l}$$

where:

v = volume of acetone used (ml)

V = volume of sample water filtered (liters)

l = path length of cuvette (cm)

665o = abs of sample at 665 nm before acidification

665a = abs of sample at 665 nm after acidification

or:

$$\text{Chlorophyll (mg/m}^3\text{)} = C/V$$

where:

C (chlorophyll a) = 11.6 (A₆₆₅) - 1.31 (A₆₄₅) - 0.14 (A₆₃₀)

C (chlorophyll b) = 20.7 (A₆₄₅) - 4.34 (A₆₆₅) - 4.42 (A₆₃₀)

C (chlorophyll c) = 55 (A₆₃₅) - 4.64 (A₆₆₅) - 16.3 (A₆₄₅)

V = volume of sample water filtered (liters)

IV. TROUBLESHOOTING / HINTS

Discussed in Methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

Appropriate comparisons of mean values between stations and groups of stations can be made in an ANOVA framework, after ensuring that the assumptions of normality and homogeneity of variance are met.

¹ Formulas preferred by author.

**UNH, JEL STANDARD OPERATING PROCEDURE
WATER SAMPLE FILTRATION AND ANALYSIS OF
TOTAL SUSPENDED SOLIDS, CHLOROPHYLL AND
PHAEOPIGMENTS**

JEL SOP 1.06
Revision 0
January 1992

VI. REFERENCES

Strickland, J. D. H. and T. R. Parsons. 1968. A Practical Handbook of Seawater Analysis. Fisheries Research Board of Canada, Ottawa. pp. 185-194.

**UNH, JEL STANDARD OPERATING PROCEDURE
ANALYSIS OF SEAWATER SAMPLES FOR AMMONIUM
(NH₄⁺) USING WET CHEMISTRY PROCEDURE**

**JEL SOP 1.07
Revision 0
January 1992**

POINT OF CONTACT:

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I. OBJECTIVE

This SOP describes the wet chemistry procedure for determining the concentration of ammonium (NH₄⁺) in seawater and estuarine water. Automated methods of analysis may also be used, but they should be EPA approved, and some results should be supported by this wet chemistry method. See JEL SOP 1.05 and 1.06 for sample collection and preparation.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Equipment

- dry HCl washed test tubes
- Auto pipettor with enough 5 ml disposable pipette tips for each sample
- 5 ml glass pipettes for standards
- 2 autopipettors (0.5 or 1 ml with 0.1 ml increments)
- test tube rack
- volumetric flasks - 2 × 100 ml, 2 × 200 ml, 250 ml, 500 ml, 1000 ml
- Beckman model 35 dual beam spectrophotometer
- a pair of 1 cm quartz cuvettes

B. Reagents

Reagent A - add 19.5 ml (17.5 g) analytical grade phenol and 0.2 g sodium nitroprusside dihydrate to 500 ml ammonia free water (deionized). Store in a dark, plastic bottle at 5°C. Should be stable for weeks, color should be slightly yellow. If it turns a green color, discard and make fresh reagent.

Reagent B - Add 140 g trisodium citrate and 11 g sodium hydroxide to 400 ml of ammonia free water. Add 28 ml fresh 5.25% sodium hypochlorite (Clorox) to this solution and dilute to 500 ml with deionized water. Store cold in a dark bottle. Should be stable for several weeks.

**UNH, JEL STANDARD OPERATING PROCEDURE
ANALYSIS OF SEAWATER SAMPLES FOR AMMONIUM
(NH₄⁺) USING WET CHEMISTRY PROCEDURE**

JEL SOP 1.07
Revision 0
January 1992

Standards:

- 0.200 M - dilute 2.67 g NH₄Cl to 250 ml with deionized water in a volumetric flask
- 2000 μM - dilute 1.0 ml of 0.200 M NH₄Cl to 100 ml in a volumetric flask
- 2 μM - dilute 1.0 ml 2000 μM to 1000 ml in a volumetric flask
- 4 μM - dilute 1.0 ml 2000 μM to 500 ml in a volumetric flask
- 10 μM - dilute 1.0 ml 2000 μM to 200 ml in a volumetric flask
- 20 μM - dilute 2.0 ml 2000 μM to 200 ml in a volumetric flask
- 40 μM - dilute 2.0 ml 2000 μM to 100 ml in a volumetric flask

III. METHODS

1. Acid clean all glassware in 10% HCl and rinse 5 times with deionized water.
2. Use standard laboratory safety procedures (protective clothing, exhaust hood) when handling reagents.
3. Prepare reagents in an exhaust hood. Keep samples covered in a hood while developing.

General Procedures:

1. Syringe 5 ml of sample into test tube using pipettor with disposable tip (see Note 2).
2. Add 0.3 ml of reagent A using autopipettor, shake.
3. Add 0.3 ml of reagent B using separate autopipettor, shake (see Note 3).
4. Using deionized water in place of sample, do steps 1-3.
5. Follow steps 1-3 using standards 2, 4, 10, 20, and 40 μM NH₄Cl.
6. Let reaction proceed at least 4 hrs in the dark, overnight is best.
7. Calibration
 - a. Set spectrophotometer to measure absorbance at 630 nm.
 - b. Using distilled water instead of sample, follow steps 1-3, 5. Measure absorbance immediately. This value will be (A_w) or reagent blank.
 - c. Read abs at 630 nm for water blank (step 4). This value will be (A_w).

**UNH, JEL STANDARD OPERATING PROCEDURE
ANALYSIS OF SEAWATER SAMPLES FOR AMMONIUM
(NH₄⁺) USING WET CHEMISTRY PROCEDURE**

**JEL SOP 1.07
Revision 0
January 1992**

8. Read absorbances for standards. Plot concentration of ammonium vs. absorbance; concentration = y axis, absorbance = x axis. Calculate a regression equation and a correlation coefficient (r) using least squares analysis.
9. Read absorbances for samples, (A_s).
10. Calculate the adjusted sample value (A_c) for samples where
$$A_c = A_s - A_{rb} - A_w.$$
11. Calculate concentration of ammonium using the regression equation from step 8 and adjusted sample value (A_c).

Notes:

1. Use test tubes for ammonium analysis.
2. All samples may be prepared first and then reagents added.
3. Reagent A may be added to all samples, then all samples can be shaken. Same procedure for Reagent B.
4. Spectrophotometer is zeroed using water in both cuvettes.
5. Range of detection is 0.4 to 40 μM.
6. Samples should be shaken before being dispensed into test tubes, and before being placed into cuvette for absorption reading.

IV. TROUBLE SHOOTING / HINTS

See Methods and Notes.

V. STATISTICAL ANALYSIS AND DATA USAGE

Calculate the sample concentration from least squares regressions of standard concentrations (x) and their absorbances (y). Calculate means of duplicate replicates and report as μM ammonium concentrations.

VI. REFERENCES

Koroleff, F. 1976. Determination of ammonia. *In*: (K. Grasshoff, ed.) *Methods of Seawater Analysis*. Verlag Chemie, NY. pp 126-133.

**UNH, JEL STANDARD OPERATING PROCEDURE
ANALYSIS OF SEAWATER SAMPLES FOR PHOSPHATE
(PO_4^{3-}) USING WET CHEMISTRY PROCEDURE**

JEL SOP 1.08
Revision 0
January 1992

POINT OF CONTACT:

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I. OBJECTIVE

This SOP describes the wet chemistry procedure for determining the concentration of phosphate (PO_4^{3-}) in seawater and estuarine water. Automated methods of analysis may also be used, but they should be EPA approved, and some results should be supported by this wet chemistry method. See JEL SOP 1.05 and 1.06 for sample collection and preparation.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Equipment

- Dry, HCl washed 125 ml Erlenmeyer flasks
- 50 ml graduated cylinder or pipette for sample
- 5 ml autopipette for dispensing reagent
- 10 ml volumetric pipette
- 200 ml volumetric flask
- 10 cm spectrophotometer cell
- Perkin-Elmer Coleman 55 Spectrophotometer

B. Reagents

Ammonium Molybdate Solution - Dissolve 15 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in 500 ml distilled water. Store in a dark bottle without sun. This is stable indefinitely. Use as long as solution is clear. Refrigerate.

Sulfuric Acid Solution - Add 140 ml concentrated H_2SO_4 to 900 ml distilled water (or 62 ml acid to 400 ml water). Store in a glass bottle. Always add acid to water. Use the hood with window down, fan on, and wear gloves.

Ascorbic Acid Solution - Dissolve 5.4 g ascorbic acid in 100 ml distilled water. Store in small plastic bottles in 20 ml. amounts, frozen.

UNH, JEL STANDARD OPERATING PROCEDURE
ANALYSIS OF SEAWATER SAMPLES FOR PHOSPHATE
(PO_4^{3-}) USING WET CHEMISTRY PROCEDURE

JEL SOP 1.08
Revision 0
January 1992

Potassium Antimonyl-Tartrate Solution - Dissolve 0.34 g $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$ in 250 ml distilled water - this may be warmed to help dissolve. The solution is stable for months and should be stored in a glass bottle. Use as long as solution is clear.

Mixed Reagent - Use 10 ml ammonium molybdate solution + 25 ml sulfuric acid solution + 10 ml ascorbic acid solution + 5 ml antimony solution or some multiple of the above (these volumes are sufficient for 10 samples). Prepare for immediate use; don't store for more than 6 hours or the solution will yellow due to ascorbic acid. Use the same graduated cylinder to eliminate error. If reagent turns at all blue or bluish green, discard.

C. Standards

Phosphate Solutions -

6000 μM : Dissolve 0.816 g anhydrous KH_2PO_4 in water and dilute to 1 L. Store in a dark plastic bottle with 1 ml chloroform in the refrigerator where it will be stable for many months. If there is any doubt that the KH_2PO_4 is dry, place in oven for at least 10 hours at 110°C .

60 μM : Dilute 10 ml 6000 μM KH_2PO_4 to 1 L. Store in a dark plastic bottle with 1 ml chloroform in the refrigerator. Replace every 10 days.

3 μM - for F factor: Dilute 25 ml 60 μM KH_2PO_4 to 500 ml (10 ml in 200 using glass pipette, in a 200 ml volumetric flask). This may be stored overnight only if refrigerated with 1 ml chloroform.

III. METHODS

1. Let the water samples warm up to room temperature.
2. Prepare the appropriate amount of mixed reagent based upon number of samples.
3. Prepare two reagent blanks using 50 ml distilled water each.
4. Prepare three 3 μM PO_4^{3-} standards using the 60 μM standards and diluting as described above. Pour 50 ml into each of three flasks.
5. Measure 50 ml of each water sample into Erlenmeyer flask (with one replicate per sample).
6. Pipette 5 ml mixed reagent into each sample, reagent blank, and standard.

**UNH, JEL STANDARD OPERATING PROCEDURE
ANALYSIS OF SEAWATER SAMPLES FOR PHOSPHATE
(PO_4^{3-}) USING WET CHEMISTRY PROCEDURE**

JEL SOP 1.08
Revision 0
January 1992

7. Wait 10 minutes, and measure absorbances at 660 nm.

Notes:

1. Let the spectrophotometer warm up for an hour before use.
2. Absorbance limitation for cells: 0.005 for 10 cm cell, 0.125 ± 001 for 4 cm cell.
3. The spectrophotometer is zeroed using water as the reference.
4. F factor is calculated using the absorbance of the standards and reagent blanks.

A_{rb} : Reagent blank absorbance - average of 2

A_s : standards $3 \mu\text{M PO}_4^{3-}$ absorbance - average of 3

A_r : sample absorbance

F: F factor: $3 \mu\text{M}/(A_s - A_{rb})$

C: Concentration in $\mu\text{M PO}_4^{3-}$, $C = F \times (A_s - A_{rb})$

5. Range of detection is 0.03 to 5 μM .
6. Samples should be shaken before being dispensed into flasks, and before being placed into cell for absorption reading.

IV. TROUBLE SHOOTING / HINTS

See Methods and Notes.

V. STATISTICAL ANALYSIS AND DATA USAGE

Appropriate comparisons of mean values between stations and groups of stations can be made in an ANOVA framework, after ensuring that the assumptions of normality and homogeneity of variance are met.

VI. REFERENCES

Strickland, J. D. H. & T. R. Parsons. 1968. A Practical Handbook of Seawater Analysis. Fisheries Research Board of Canada, Ottawa. pp 52-55.

UNH, JEL STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF *Clostridium perfringens* IN
MARINE SEDIMENTS

JEL SOP 1.09
Revision 0
January 1992

POINT OF CONTACT:

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I. OBJECTIVE

Clostridium perfringens is a spore-forming bacterium that is a normal inhabitant of the intestinal tract of humans and other animals, and is also commonly found in both soil and water. *C. perfringens* is a pathogen responsible most commonly for the symptoms of gangrene, as well as food poisoning, profuse diarrhea, and small bowel infections. *C. perfringens* has been used as an indicator of the sanitary quality of water, shellfish and sediments. The longevity of the endospore allows for long-term studies of fecal contamination, but also makes *C. perfringens* less useful for routine determination of recent fecal contamination. The most probable number method is commonly used as a means of enumerating *C. perfringens* in marine sediments and shellfish.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Glassware

- Blender (sterilized at 121°C for 15 minutes)
- 1 liter polypropylene bottles
- 20 × 150 mm test tubes
- 10 ml sterile pipettes

B. Media and Diluent

Media Ingredients:

- Homogenized milk
- Iron filings (Klinger-fine grain)

Diluent Ingredients:

- Sodium chloride (Baker Analyzed)
- Phosphate buffer (Baker Analyzed)
- Deionized water

C. Equipment

- Waterbath (45°C)
- Analytical balance

**UNH, JEL STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF *Clostridium perfringens* IN
MARINE SEDIMENTS**

JEL SOP 1.09
Revision 0
January 1992

- Blender
- Test tube mixer (vortex type)

III. METHODS

A. Media Preparation: Iron Milk Media

1. Weigh 0.2 g of iron filings into each of fifteen test tubes.
2. Add 10.0 ml of homogenized milk to each test tube.
3. Autoclave at 116°C for 10 minutes.
4. Temper the media to 45°C prior to assay.

B. Diluent Preparation: Phosphate Buffered Saline (PBS)

1. Weigh 8.5 g of sodium chloride into a polypropylene bottle.
2. Add 1.0 liter of deionized water and stir to dissolve.
3. Add 1.25 ml of standard phosphate buffer.
4. Autoclave at 121°C for 15 minutes.
5. Store at room temperature for up to one month.

C. Sample Preparation

1. The examiner should wear disposable plastic gloves with surfaces disinfected with isopropyl alcohol.
2. Aseptically collect subsamples from sediment samples and weigh into a dilution tube or a blender containing PBS of appropriate volume to give the desired dilution.
3. Vortex the sample in the dilution tube or blend in a Waring blender for 1 minute.
4. Use prepared samples immediately.

UNH, JEL STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF *Clostridium perfringens* IN
MARINE SEDIMENTS

JEI SOP 1.09
Revision 0
January 1992

5. Dry triplicate sediment aliquots at 105°C to determine % dry weight of sediment.

D. Sample Analyses

1. Inoculate each of 5 iron milk tubes with 10.0 ml of the prepared sample
2. Inoculate each of 5 iron milk tubes with 1.0 ml of the prepared sample.
3. Inoculate each of 5 iron milk tubes with 1.0 ml of a 10:1 dilution of the prepared sample.
4. In order to avoid indeterminate results (all positive tubes), inoculate sets of 5 tubes with 1.0 ml of 10⁻² and 10⁻³ dilutions of the prepared sample.
5. Incubate tubes at 45°C for 18 hours.
6. Examine tubes for the presence of stormy fermentation.
7. Score positive tubes (those with stormy fermentation) for *Clostridium perfringens*.

E. Using the Most Probable Number Geometric Series

1. Count the number of positive iron milk tubes from each of the five dilutions performed. Choose the highest dilution that gives five positive tubes and the two succeeding higher dilutions. Use the results of three dilutions to form a 3 digit code. Locate your three digit code on the most probable number geometric series chart. Read the MPN number which corresponds to your three digit code. Compute the MPN value of *C. perfringens* per 100 ml of sample by factoring in sample volumes used compared to sample volumes indicated in MPN table. For example, if 5 tubes containing 10.0 ml sample, 2 tubes containing 1.0 ml of sample and 1 tube containing 0.1 ml of sample were positive, the three digit code would be 521. The MPN number for a code of 521 in dilutions containing 10.0, 1.0 and 0.1 ml sample volumes is 70. The density of *C. perfringens* per 100 ml of sample would be 70. If the dilutions were 0.1, 0.01 and 0.001 ml with a 3 digit code of 521, the density of *C. perfringens* per 100 ml of sample would be 7000.
2. Use measured % dry weight (D.W.) of sediment and initial sample dilution factor to convert the MPN value to *C. perfringens* per 100 g D.W. sediment.

**UNH, JEL STANDARD OPERATING PROCEDURE
MOST PROBABLE NUMBER METHOD FOR THE
ENUMERATION OF *Clostridium perfringens* IN
MARINE SEDIMENTS**

JEL SOP 1.09
Revision 0
January 1992

IV. TROUBLE SHOOTING / HINTS

Samples and dilutions of samples must be properly mixed prior to addition to enrichment tubes.

V. STATISTICAL ANALYSIS AND DATA USAGE

Tabulate and summarize the data. Data are used in conjunction with other indicator tests to determine sanitary quality.

VI. REFERENCES

- Calise, B. A. 1991. ERL-N Standard Operating Procedure. Most Probable Number Method for the Enumeration of *Clostridium perfringens* in Marine Waters. ERL-N SOP 1.03.017, 4 pp.
- St. John, W. D., J. R. Matches and M. M. Wekell. 1982. Use of iron milk medium for enumeration of *Clostridium perfringens*. *J. Assoc. Of. Anal. Chem.* 65:1129-1133.

**UNH, JEL STANDARD OPERATING PROCEDURES
SEDIMENT CORING, CORE EXTRUSION AND
SUBSAMPLING**

JEL SOP 1.10
Revision 0
January 1992

POINT OF CONTACT:

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I. OBJECTIVE

Collect and sample sediment cores for textural, bacteriological or toxicological analyses.

II. NECESSARY MATERIALS AND EQUIPMENT

- Research vessel with adequate winches for handling gravity corer (at least 150 kg capacity)
- Benthos Gravity Corer or equivalent with transparent liners, end caps, stainless steel core catchers. PVC check valve and manganese bronze nose cone.
- Appropriate clothing and protection for sampling in contaminated sites (rubber boots, raingear - jacket and pants, rubber gloves, glasses)
- Cooler or plastic garbage barrel with ice
- Duct tape
- Core extruder - rubber stopper attached to wood pole
- Core tray
- Aluminum weighing dishes
- Teflon or plastic knives
- Knife, piano wire or scalpel

III. METHODS

A. Coring

1. Prior to use, wash each component of the coring system which will come in contact with any samples with soapy water and thoroughly rinse with tap water. In addition, rinse the equipment with ambient water between uses in the field. Cut the core liner into a 1.6 m and 1.0 m section prior to coring and unite with duct tape. Place the 1.0 m section on top.
2. When on station first take a grab sample to determine the suitability of the bottom for gravity coring. Rocky bottoms will damage the coring apparatus while sandy bottoms are very hard to penetrate. Soft, muddy bottoms are most suitable.

**UNH, JEL STANDARD OPERATING PROCEDURES
SEDIMENT CORING, CORE EXTRUSION AND
SUBSAMPLING**

**JEL SOP 1.10
Revision 0
January 1992**

3. Assemble the core liner, core catcher and nose cone in the gravity corer. Lift the corer to the desired height and allow to either free fall or lower to the bottom with resistance on the winch system (depending on desired penetration into the sediment). The more rapid fall velocities allows more penetration; however, more disturbance of the sediment may occur. Make every effort to minimize the disturbance of the surface of the sediment during the coring procedure. This is accomplished by controlling the fall velocity of the corer.
4. Retrieve core from bottom using the winch system, and raise out of the water.
5. Leave the gravity corer in the vertical position while removing the core liner (with sediment) from the gravity corer barrel. Remove the nose cone from the core barrel and place a plastic cap on the bottom of the core liner (tape in place). Remove the upper 1 m section of the core liner by cutting the duct tape and allowing the water to drain. The sediment will normally be below the junction of the two lengths of core liner. If it is not, then the core liner must be left united. Normally, the core catcher is left in the barrel until the core is brought back to the laboratory. If the core catcher needs to be removed in the field, siphon off the water above the sediment, hold a plug at the base of the core to stop the sediment from sliding downward, remove the core catcher and recap the core liner.
6. After recovery of the sediment core (still in the core liner), maintain the core in a vertical position while the water above the sediment surface is siphoned off. Cap the top of the 1.6 m section of the core containing the sediment, place the base of the core in a large cooler with ice and cover the entire core with a opaque shielding. Maintain the core in a vertical position until analyzed.
7. Store the sediment core at $\sim 4^{\circ}\text{C}$ in the dark in a vertical position until sampled in the laboratory. The cores should be extruded and subsampled as soon as possible. Samples for textural analyses can be stored for relatively long periods as long as the sediments do not dry out. However, samples for toxicological analysis should be processed relatively quickly. A maximum holding time of 2 weeks was recommended for sediments for toxicological analyses (Puget Sound Estuary Program 1991).

B. Core Extrusion

This sequence assumes that the core has had the water siphoned off and that the core has been chilled to $\sim 4^{\circ}\text{C}$. However, the chilling is not mandatory. It serves to maximize the cohesiveness of the sediment for ease in handling.

**UNH, JEL STANDARD OPERATING PROCEDURES
SEDIMENT CORING, CORE EXTRUSION AND
SUBSAMPLING**

JEL SOP 1.10
Revision 0
January 1992

1. Place the core gently in a horizontal position and remove the caps. Be sure that the surface does not slump.
2. Extrude the core by using a plunger to push the core out of the tube (from the bottom) onto a core tray being careful to not disturb the sediment. The core may also be extruded by tilting the core liner gently and allowing the core to slide out the bottom of the liner onto the core tray.
3. The core is now ready for description and subsampling. Be sure to remove the outside of the sediment sample where contact was made with the core liner.
4. Remove the surface layer of the core with a scalpel, sharp knife or piano wire. Subsamples can be removed with the same apparatus. However, if toxicological, trace metal, etc. analyses are to be done, use a teflon or plastic knife.

C. Core Subsampling

This procedure assumes that the core has been extruded into a core tray.

1. Determine desired sampling depths. At each depth remove 2 to 3 cc of sediment. Analyze for moisture and combustible content as described in the section on Sediment Textural Analysis. Subsequently, remove the appropriate size samples for textural, microbiological or toxicological analyses and place in sterile, appropriately cleaned plastic bags. The samples are then frozen or stored in a refrigerator until analyzed.

IV. TROUBLE SHOOTING / HINTS

A. Coring

The main problem that may arise is the substrate is too hard to allow penetration by the gravity corer due to coarse sediments or bedrock. It is not advisable to attempt coring if the substrate is very coarse as the nose cone may be damaged. In the event that a gravity core can not be taken, it may be possible to take suitable cores in shallow depths with a vibra-coring system or by forcing a core barrel directly into the sediment.

B. Extrusion

Care must be taken to be certain that the surface or bottom of the core does not slump during extrusion. This can be accomplished by making a temporary cap for the top or bottom with a stopper with the appropriate diameter. After the core has been

**UNH, JEL STANDARD OPERATING PROCEDURES
SEDIMENT CORING, CORE EXTRUSION AND
SUBSAMPLING**

**JEL SOP 1.10
Revision 0
January 1992**

extruded and is in the core tray, examine the sediment carefully looking for any indications of disturbance to the core and note them. Things to look for include smearing, bioturbation, slumping, or materials being dragged down the side of the barrel (e.g. eelgrass). This is only a partial list. If the core has been disturbed, it may not be suitable for analysis.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not applicable.

VI. REFERENCES

Puget Sound Estuary Program. 1991. Recommended Guidelines for Conducting Laboratory Bioassays on Puget Sound Sediments (Interim Final). Prepared for U.S. Environmental Protection Agency, Region 10, Office of Puget Sound, Seattle, WA.

**UNH, JEL STANDARD OPERATING PROCEDURE
SEDIMENT TEXTURAL ANALYSIS**

JEL SOP 1.11
Revision 0
January 1992

POINT OF CONTACT:

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I. OBJECTIVE

This SOP describes a procedure for determination of grain size statistics, moisture content and combustible content (as a measure of particulate organics) of a sediment sample in order to characterize the sediments.

II. NECESSARY MATERIALS AND EQUIPMENT:

- Standard set of sieves, sieve shaker and wet sieves (63 μ and 2 mm)
- Graduated cylinders (1 L), 20 ml pipette, 50 ml beakers, 1 L beakers, stirring rod
- Dispersant (sodium hexametaphosphate - Calgon)
- 30% Hydrogen peroxide (H_2O_2) or equivalent oxidizer
- Laboratory drying oven
- Muffle furnace
- Balance accurate to 0.0001 g
- Deionized water
- Aluminum weighing dishes
- Squeeze bottles
- Funnel (greater than 20 cm diameter)
- Stirring rod for a 1 L graduated cylinder
- Desiccator

III. METHODS

A. Moisture And Combustible Content

1. Place 2 to 3 cc of sample in a preweighed aluminum dish and weigh to the nearest 0.0001 g to determine the wet weight. Subsequently, dry in an oven at 50-75°C for 24 hrs or until completely dry. Remove from oven and cool in a desiccator to room temperature. Place sample in weighing room for about 1 hr or until the sample stops gaining weight due to ambient air moisture. Reweigh to determine the moisture content - the % weight loss ((wet weight - dry weight) / wet weight). Next, heat sample in muffle furnace at 450°C for 4 hrs

to determine combustible content - the % weight loss after heating ((dry weight - combusted weight) / dry weight). Again, after removing the sample from the oven, place in a desiccator until cooled to room temperature, then place in ambient air until weight is stable.

B. Size Analysis: Wet Sieving

1. Place the sample in a large beaker (1 L) and add approximately 20-30 ml of 30% H_2O_2 . Add a small amount of H_2O_2 at a time to be sure the sample does not foam over the top of the beaker (~10-15 ml initially, then 10-15 ml after 24 hours, etc.). The amount of sediment sample to use depends on the analyses to be run. Approximately 10 - 15 g dry weight is needed for pipette analysis and 30 - 70 g for sieve analysis. Stir to break up the sample.
2. After as much of the organics as possible have been removed as indicated by lack of bubbling upon adding more H_2O_2 and the remaining liquid has been decanted, remove the sea salts which may be in the sample. This is done by adding deionized water, stirring and decanting. Place the sample in a bottle and fill with dispersant (~0.2 g/L of Calgon in deionized water). Stir vigorously to be certain that the sediment particles are separate. If the sample appears to have gravel particles (greater than 2 mm), wet sieve the sample through a 2 mm stainless steel sieve to remove the gravel. Rinse the gravel with deionized water into a preweighed beaker, dry and set aside (to determine % gravel).
3. Wet sieve the sample through a 63 μ stainless steel sieve. This is done by placing the wet sieve in a plastic funnel of slightly larger diameter which drains into a 1 L graduated cylinder. Place the sample on the wet sieve and gently rinse all the sediment finer than 63 through the sieve with dispersant in a squeeze bottle. Be careful not to touch the sieve with your hand or anything that will damage the mesh.
4. Wash the material on the wet sieve into a preweighed beaker using deionized water and dry. This is the sand fraction (63 to 2 mm). The material in the graduated cylinder is the mud fraction (silt and clay).

C. Sieve Analysis of the Sand Fraction

1. Weigh the dry and disaggregated sand fraction to the nearest 0.01 g.
2. Select a set of sieves that cover the range of sediment sizes to be analyzed and place the screens in order with the coarsest at the top and the pan on the

**UNH, JEL STANDARD OPERATING PROCEDURE
SEDIMENT TEXTURAL ANALYSIS**

JEL SOP 1.11
Revision 0
January 1992

bottom. Use a maximum of $\frac{1}{2}$ phi intervals. Pour the sand sample on the top sieve and place in the sieve shaker for 15 minutes.

3. Carefully remove the sediment from each sieve and weigh to 0.01 g.
4. Total the weight of the sand in each size interval (sieve). If the weight is not within 2% of the original sediment weight, then rerun the analysis.
5. If the analysis is okay, then proceed with determining the size frequency distribution and graphic grain size statistics (mean size, sorting, skewness and kurtosis) as outlined in Folk (1980).
6. Add any sediment that passes through the 63μ sieve during the shaking processes to the fine fraction to be used for the pipette analysis.

D. Pipette Analysis of the Silt and Clay (Mud) Fraction

1. Add dispersant (Calgon) to the mud fraction until the graduated cylinder has exactly 1 L in it. Thoroughly mix with stirring rod for approximately 2 to 5 minutes being very careful not to splatter any of the sample. Accurately determine the amount of dispersant added to the sample as this has to be subtracted out during the statistical analysis. This can be done by mixing a large quantity of dispersant, removing 20 ml samples with a pipette, drying and determining dispersant weights.
2. Allow the sample to stand overnight to see if any flocculation occurs. If the sample flocculates, the sample preparation has to be redone or additional dispersant added.
3. If the sample is suitable for analysis, restir for 2 to 5 minutes with a stirring rod or with a mechanical stirrer. Be sure to stir the length of the column.
4. The moment the stirring is stopped is time = 0 and the analysis has begun. At the prescribed time intervals and depths outlined in Folk (1980), carefully pipette out 20 ml samples from the graduated cylinder and place in preweighed (to 0.0001 g) 50 ml beakers or equivalent. Take the appropriate samples to determine the amount of sediment in each size interval from 4 to 14 phi as described in Folk (1980). Normally, the 10 - 14 phi interval is determined by computation.
5. Dry the sediment in each beaker 50-75°C for a minimum of 24 hours or until completely dry. After drying, place the beakers initially in a desiccator until the

**UNH, JEL STANDARD OPERATING PROCEDURE
SEDIMENT TEXTURAL ANALYSIS**

JEL SOP 1.11
Revision 0
January 1992

sample is at room temperature, then place in the weighing room until the samples are at equilibrium with ambient humidity and reweigh.

IV. TROUBLE SHOOTING

Included in Methods.

V. STATISTICAL ANALYSIS AND DATA USAGE

1. Determine the size frequency distribution and graphic statistics (mean, sorting, skewness and kurtosis) using the methods outlined in Folk (1980). If both sieve and pipette analyses were done for a sample, then combine the data sets to determine a single set of statistics (Folk 1980).

VI. REFERENCES

Folk, R. L. 1980. Petrology of Sedimentary Rocks. Hemphill Publishing Company, Austin, Texas. 185 pp.

UNH, JEL STANDARD OPERATING PROCEDURE
ANALYSIS OF SEAWATER SAMPLES FOR NITRATE
AND NITRITE (NO_3^- , NO_2^-) USING AN AUTOMATED
PROCEDURE

JEL SOP 1.12
Revision 0
March 1992

POINT OF CONTACT:

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I. OBJECTIVE

This SOP describes the automated chemistry procedure for determining concentrations of nitrate and nitrite (NO_3^- , NO_2^-) in seawater and estuarine water. The automated analyzer used is a flow-injection system manufactured by Lachat, Instruments, which supplies EPA-approved methodologies. Wet chemistry methods based on similar analyses (also EPA-approved) should be performed on some samples to verify the automated output. See JEL SOP 1.05 and 1.06 for sample collection and preparation.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Equipment

- LACHAT QuickChem Automated Ion Analyzer
- Auto sampler
- Erlenmeyer flasks for reagents
- Pipettors and pipette tips for standards
- Volumetric flasks for preparation of standards and reagents

B. Reagents

50% Artificial Seawater Carrier and Standards Diluent - To 1 liter deionized water, add 14.65 g sodium chloride (NaCl), 4.7 g magnesium sulfate (MgSO_4) and 0.1 g sodium bicarbonate (NaHCO_3). Swirl until dissolved.

Ammonium Chloride buffer, pH 8.5 - In a 1 liter volumetric flask, dissolve 85 g ammonium chloride (NH_4Cl), and 1 g disodium ethylenediamine tetraacetic acid dihydrate ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$) in ca. 800 ml water. Adjust the pH up to 8.5 with 15 M sodium hydroxide, and bring to volume.

15 M Sodium hydroxide - Add 150 g NaOH slowly to 250 ml water. Swirl until dissolved and store in a plastic bottle.

UNH, JEL STANDARD OPERATING PROCEDURE
ANALYSIS OF SEAWATER SAMPLES FOR NITRATE
AND NITRITE (NO_3^- , NO_2^-) USING AN AUTOMATED
PROCEDURE

JEL SOP 1.12
Revision 0
March 1992

Sulfanilamide color reagent - To a 1 liter volumetric flask add about 600 ml water. Then add 100 ml 85% phosphoric acid (H_3PO_4), 40 g sulfanilamide and 1 g N-(1-naphthylethylenediamine dihydrochloride (NED). Shake to wet, and stir to dissolve (30 min) and bring to volume. Dilute to the mark, and invert three times. Stored in a dark bottle, the solution is stable for one month.

C. Standards:

Instructions are given for nitrate standards with instructions specific for nitrite standards given in bold in parentheses. Make nitrite working standards daily; the stock is stable for only 3 to 5 days.

Stock nitrate (nitrite) standard - 10.7 M - In a 500 ml volumetric flask dissolve 0.54 g potassium nitrate (**0.455 g potassium nitrite**) in about 300 ml 50% artificial seawater. Add 1 ml chloroform. Dilute to the mark with 50% artificial seawater and invert 3 times.

Concentrated standards - 107 μM - In a 500 ml volumetric flask add 5 ml stock nitrate (**nitrite**) standard. Dilute to the mark with 50% artificial seawater and invert 3 times.

Sets of three working standards - Use 107 μM concentrated standards to make each set:

10.7 μM - 5 ml in 50 ml volumetric flask. Dilute to mark with 50% artificial seawater.

4.28 μM - 2 ml in 50 ml volumetric flask. Dilute to mark with 50% artificial seawater.

2.14 μM - 1 ml in 50 ml volumetric flask. Dilute to mark with 50% artificial seawater.

III. METHODS

Samples will be analyzed for nitrate+nitrite by running sample through a cadmium column (where all nitrate becomes reduced to nitrite). Nitrite alone is determined using the system after the cadmium column has been disconnected. Nitrate alone can be calculated if the efficiency of the cadmium column is determined (Step 5).

1. Acid clean all glassware in 10% HCl and rinse 3 times with deionized water.
2. Use standard laboratory safety procedures (protective clothing, exhaust hood) when handling reagents.

**UNH, JEL STANDARD OPERATING PROCEDURE
ANALYSIS OF SEAWATER SAMPLES FOR NITRATE
AND NITRITE (NO_3^- , NO_2^-) USING AN AUTOMATED
PROCEDURE**

JEL SOP 1.12
Revision 0
March 1992

3. Prepare appropriate amounts of standards and reagents.
4. Set up autoanalyzer with reagents, carrier, and deionized water flowing through manifold. After reagents have run through, attach cadmium column to the manifold board.
5. Calculate column efficiency with the column in line:
 - a. Load the 3 working standards (in descending order of concentration) of nitrite and nitrate.
 - b. Download Nitrate method from the computer software to the autoanalyzer. (Follow the Lachat instruction manual and QuikChem Method to define method.)
 - c. Complete sample identification and submit sample tray.
 - d. Check calibration of nitrite standards. If they pass approval ($r > 0.95$ for each of five peak chords; standard deviation $< 50\%$ of slope), record slope.
 - e. Check calibration of nitrate standards. If they pass approval, record slope.
 - f. Column efficiency = nitrate slope/nitrite slope. If column efficiency is greater than 0.80, note and continue. If not, replace the cadmium column.
6. Remove nitrite working standards and load the samples into the tubes on the auto sampler tray.
7. Complete sample identification and replicate commands and submit sample tray.
8. Recheck calibration of standards. If it passes approval, run the samples.
9. Replace nitrate standards with nitrite standards and repeat steps 7 and 8.

**UNH, JEL STANDARD OPERATING PROCEDURE
ANALYSIS OF SEAWATER SAMPLES FOR NITRATE
AND NITRITE (NO_3^- , NO_2^-) USING AN AUTOMATED
PROCEDURE**

JEL SOP 1.12
Revision 0
March 1992

IV. TROUBLE SHOOTING / HINTS

If calibration of standards fails, rerun standards. If failure reoccurs, check standard preparation procedure, range of standards (to insure Beer's Law applies), and remake standards.

If sample salinity is less than 5 ppt, standards should be made with 10% artificial seawater, and carrier replaced with 10% artificial seawater (instead of 50% artificial seawater).

V. STATISTICAL ANALYSIS AND DATA USAGE

Tabulate the sample concentrations generated from least squares regressions of standard concentrations (x) and their absorbances (y) by the software. Subtract nitrite concentrations divided by the column efficiency from nitrate+nitrite concentrations to obtain nitrate concentrations ($\mu\text{M nitrate} = [\mu\text{M nitrate} + \text{nitrite}] - [\mu\text{M nitrite}/\text{column efficiency}]$). Calculate means of duplicates (if run) and replicates and report as μM nitrate and nitrite concentrations.

VI. REFERENCES

Lachat Instruments. 1991. QuikChem Method #30-107-04-1-A Nitrate/Nitrite, Nitrite in Brackish Waters. Lachat Instruments, Milwaukee, WI.

U.S. Environmental Protection Agency. 1983. Methods for Chemical Analysis of Water and Wastes, EPA-600/4-79-020, Rev. 3/83, Method 353.2. U.S. EPA, Washington, DC.

**NAI STANDARD OPERATING PROCEDURE
WINTER FLOUNDER (*Pleuronectes americanus*) AND
LOBSTER (*Homarus americanus*) COLLECTION FOR
CHEMICAL ANALYSIS**

JEL SOP 1.13
Revision 0
March 1992

POINT OF CONTACT:

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I. OBJECTIVE

Enumeration and collection of winter flounder and lobster using trawls and subsequent excision and preparation of specific organs for chemical tissue analysis.

II. NECESSARY MATERIALS AND EQUIPMENT

- Fishing or research vessel equipped with otter trawl rig
- Protective clothing
- Data sheets
- Strong plastic bags for freezing and storing tissue
- Labeling pens
- Scale ($\pm 1g$)
- Filet knives
- Meter sticks

III. METHODS

A. Winter Flounder Sampling

Collect winter flounder using otter trawls; three tows at each station. Trawl for five minutes (bottom time) at a water speed of ca. 2 knots. For each station, record the number of winter flounder and collect one sample each of 300 grams of flounder flesh and 50 grams of flounder liver. Place each tissue type separately in a plastic bag and label. Store samples on ice in the dark and freeze upon return to shore. Keep samples frozen until chemical analyses.

B. Lobster Sampling

Collect lobster using three otter trawls at each station, as for the flounder collections. Tow gear for five-minutes at a water speed of ca. 2 knots. Record the number and length of each lobster caught and collect one sample each of 300 grams of lobster tail flesh and 25-50 grams of the hepatopancreas ("tomally"). Place each tissue

**NAI STANDARD OPERATING PROCEDURE
WINTER FLOUNDER (*Pleuronectes americanus*) AND
LOBSTER (*Homarus americanus*) COLLECTION FOR
CHEMICAL ANALYSIS**

JEL SOP 1.13
Revision 0
March 1992

type separately in a plastic bag and label. Samples are treated as for winter flounder (above).

IV. TROUBLE SHOOTING / HINTS

Rough bottom topography may limit the ability to sample directly on station; bathymetric maps, exploratory grab sampling, or conversations with local fishermen may indicate the closest sampling area. The number of trawls in some areas have to be increased if populations are small and cannot provide enough tissue, but organisms from only the first three tows are enumerated and measured.

V. STATISTICAL ANALYSIS AND DATA USAGE

None appropriate for tissue collections or such limited sampling of these populations.

VI. REFERENCES

None available.

**NAI STANDARD OPERATING PROCEDURE
COLLECTION OF SEDIMENT SAMPLES FOR
CHEMICAL AND TOXICOLOGICAL ANALYSES,
AND CHARACTERIZATION OF BENTHOS**

**JEL SOP 1.14
Revision 0
March 1992**

POINT OF CONTACT:

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I. OBJECTIVE

Collection of sediment samples for characterization of the benthic infauna (> 1mm).

II. NECESSARY MATERIALS AND EQUIPMENT

- Shipek or similar grab sampler and gear for deployment/retrieval
- Vessel suitable as a working platform
- Protective clothing, including gloves and glasses
- Sample containers
- Coolers with ice
- Teflon scoop
- 0.5 mm mesh screen
- Decontamination squirt bottles with nitric acid, hexane, and deionized water

III. METHODS

A. Decontamination of sampler

Prior to sampling sediments at each station, decontaminate the grab sampler to prevent cross contamination between stations. Remove gross contamination of sediments with sea water prior to the decontamination procedure. Decontaminate the grab sampler as follows: rinse with sea water; spray with nitric acid; rinse with deionized water; spray with hexane; rinse with deionized water.

B. Sediment Collection

1. Sampling for Chemical Analyses

Collect one-300 gram composite sample that is composed of sediment from four separate grab samples at each station. These samples will be analyzed for TOC, TBT, and heavy metals. Also collect a sample for grain size (JEL SOP 1.11) and Clostridium (JEL SOP 1.09) from each grab. In addition, from each of the four grabs, collect a 300 gram sample and archive. Store all sediment samples on ice in

**NAI STANDARD OPERATING PROCEDURE
COLLECTION OF SEDIMENT SAMPLES FOR
CHEMICAL AND TOXICOLOGICAL ANALYSES,
AND CHARACTERIZATION OF BENTHOS**

JEL SOP 1.14
Revision 0
March 1992

the dark and then freeze upon return to shore except for grain size and Clostridium samples which should be kept cool only. Use an external label to label each sample with appropriate identifying information.

2. Sediment Sampling for Toxicological Analyses

Skim the top 1-2 cm of sediment from each grab with a clean teflon scoop for toxicological analysis to collect a one gallon composite sample. Take extra grabs as required. Handle samples as above.

3. Sediment Sampling for Benthos

At each station, take four replicate samples using the grab sampler (0.04 m²). Bag samples with an internal label and sieve through 0.5 mm box screens. Since the objective is to record presence of benthic infauna, any macrophytes and related epifauna should be excluded. The internal tag as well as an external tag on the sample container should remain with the sample for identification purposes. Add magnesium sulfate to the container to relax the benthic fauna followed by 6% formalin to fix the samples. Rose bengal may be added later to facilitate sorting the benthic fauna from the remaining sediment.

IV. TROUBLE SHOOTING / HINTS

If the sediment is fine grained and fairly well sorted, the Shipek works well. However, if the sediment is predominantly sandy or rocky, this sampler fails and the sample must be obtained by hand if in shallow waters or by using divers if in deeper waters.

V. STATISTICAL ANALYSIS AND DATA USAGE

Not appropriate for sample collection procedures

VI. REFERENCES

Shipek sediment sampler model 860 manual. Hydro Products, Dillingham Corporation, San Diego CA.

**NAI STANDARD OPERATING PROCEDURE
SORTING AND IDENTIFICATION OF
BENTHIC INVERTEBRATES**

JEL SOP 1.15
Revision 0
March 1992

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I. OBJECTIVE

To sort and identify benthic invertebrates from sediment samples in order to characterize their benthic infaunal communities.

II. NECESSARY MATERIALS AND EQUIPMENT

A. Equipment

- Ventilated work area
- Sorting bowls or trays
- Dissecting microscope
- Forceps, dissection needles
- Shell vials
- 0.5 mm mesh screen
- Wire basket (6 mm mesh) within bucket
- Labeling pens

B. Reagents

Relaxant - 546 grams magnesium sulfate (epsom salts) per 18 liters tap water (20% solution).

Stain - 25 grams rose bengal per 2000 ml tap water.

6% buffered formalin - three parts filtered seawater to one part 25% buffered formalin (16.9 liters filtered (62 3/m 1/m) seawater, 1.1 liters 38% formaldehyde, 171 grams borax yields 18 liters 25% buffered formalin).

III. METHODS

Generally, processing of macrofaunal samples is divided into three steps with the sample being preserved after the first step: 1) gross sorting - removal of macrophytes from the sample or sieving; 2) sorting - separation according to taxa; and 3) identification - identification to the lowest taxon possible, preferably genus and species.

A. Gross Sort

Gross sorting is a quick separation of easily observed animals and macrophytes without the use of dissecting microscopes. This process should be done the same day samples are taken and can be done in the field. If the samples must wait overnight before being finished, refrigerate but do not freeze.

1. Wash the sample out of the collection container with seawater into a bucket. Check the container for any remaining animals. At the discretion of the gross sort coordinator, the sorter may "agisort" the sample. Agisort is a procedure used to separate the larger algae from the animals, clumps of algae and the sample residue. Vigorously agitate the sample in a wire basket with a 6 mm mesh within a bucket of seawater. Seive both sample fractions through the appropriate screen size, 0.5 mm, gross sorted, and then combine prior to subsequent procedures.
2. Separate and discard macrophytes.
3. Preserve animals with 6% buffered formalin and label inside and outside of jar(s) exactly as the original sample.
4. Store preserved samples in a facility where air temperatures are maintained above 10°C until the sorting process begins.

B. Sample Preservation

Following removal of macrophytes, the sample may be sieved and preserved. Samples may also be relaxed and stained.

1. Seive the sample through a 0.5 mm mesh size. Use seawater as the washing medium; samples may have been seived in the field.

**NAI STANDARD OPERATING PROCEDURE
SORTING AND IDENTIFICATION OF
BENTHIC INVERTEBRATES**

**JEL SOP 1.15
Revision 0
March 1992**

2. If samples are to be relaxed or stained, the fauna and sediments that remain on the sieve should be placed in a solution of relaxant (MgSO_4) and rose bengal stain so that the volume of solution in the container is half again as high as the sediment. Add 1 cc concentrated rose bengal solution per 16 oz. sample.
3. After 20 minutes, wash the relaxant/stain solution from the sediments through the 0.5 mm sieve.
4. Preserve the fauna and sediments in a solution of 6% formalin buffered with borax so that the volume of formalin solution is half again as high as the sediment.

C. Sorting

Sorting is the separation of animals from sediments using a dissecting microscope. The following guidelines apply:

1. Replace the formalin in all sample jars with 70% ethanol.
2. Using a dissecting microscope, separate all animals from the sediment.
3. Place the animals in the lowest category practical from those listed in Table 1.

D. Final Identification

Final identification has two objectives: 1) to identify all animals in the sample to the lowest taxon possible; and 2) to determine the number and biomass of each taxon in the sample. This process is carried out as follows:

1. Identify all animals to the lowest possible taxon, preferably genus and species, using the most current scientific literature and the verified reference collections.
2. Add problematic, new and rare species to reference collections of the laboratory or associated university.
3. Upon completion of identification, return sample to the vials, place an initialed tag in each vial to mark them as identified, and return them to the storage area.

**NAI STANDARD OPERATING PROCEDURE
SORTING AND IDENTIFICATION OF
BENTHIC INVERTEBRATES**

**JEL SOP 1.15
Revision 0
March 1992**

TABLE 1. LOWEST TAXONOMIC CATEGORIES FOR SORTING.

Porifera	Amphipoda
Hydrozoa	Isopoda
Bryozoa	Cumacea
Chordata	Caprellida
Entoprocta	Mysidacea
	Caridea
Bivalvia	Pycnogonida
Gastropoda	Decapoda
Opisthobranchia	Cirripedia
Polyplacophora	
Anthozoa	
	Asteroidea
Polychaeta	Echinoidea
Turbellaria	Ophiuroidea
Nemertea	Holothuroidea
Oligochaeta	
Sipunculida	
Hiuridinea	

4. Note the following conditions:
 - a. Heads of polychaetes must be present. Do not count headless animals.
 - b. A minimum of ten zooids are necessary to identify and record the presence of bryozoans.
 - c. For hydrozoans, the presence of the main stem and/or branches with hydranths is necessary for identification.
 - d. To identify solitary chordates, the lobes of the siphons must be visible. Record colonial chordates usually present in large quantities.
 - e. Identify porifera by examining the spicules after dissolving a part of the sponge body in bleach. Occasionally, the entire sponge colony is so small as to be dissolved during identification. Maintain a photographic record of the spicules of these sponges for QC purposes.

**NAI STANDARD OPERATING PROCEDURE
SORTING AND IDENTIFICATION OF
BENTHIC INVERTEBRATES**

**JEL SOP 1.15
Revision 0
March 1992**

- f. For molluscs, gastropods must have tissue within the shells and bivalves must be hinged with or without tissue present. If the valves break apart upon inspection or bore holes are present, do not count the animal. However, *Anomia* sp. need only have the top single valve present for enumeration. Nudibranchs and chitons must have the oral area present.
- g. Echinoderms must have a central disc or oral area present.
- h. Heads of arthropods must be present. Do not count eyes by themselves. For barnacles, the scutum and tergum must be present. Do not enumerate molts.

IV. TROUBLE SHOOTING / HINTS

Discussed in Methods, and see Items D.4.a-h.

V. STATISTICAL ANALYSIS AND DATA USAGE

Appropriate comparisons of mean values between stations and groups of stations can be made in an ANOVA framework, after ensuring that the assumptions of normality and homogeneity of variance are met.

VI. REFERENCES

Gosner, K.L. 1971. Guide to Identification of Marine and Estuarine Invertebrates. Wiley, New York.

**MESO STANDARD OPERATING PROCEDURE
ANALYSIS OF ORGANOTIN COMPOUNDS IN
WATER, SEDIMENT, AND TISSUE**

**FEBRUARY 1992
PAGE 1 OF 8**

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I. INTRODUCTION

The purpose of this document is to provide information for standardized analyses of organotin compounds in seawater, sediment and tissue and to identify the QA/QC procedures required. The sample collection and analysis procedures follow guidelines given in references 9 and 10 addressing aquatic monitoring and analysis for organotin compounds. Appendix A (reference 6) provides results of optimization techniques to obtain sub-part-per-trillion detection levels in seawater and part-per-billion detection levels in tissues and sediments.

II. SAMPLING PROCEDURES

A. Water sample collection

Water samples cannot be collected in most common water sampling devices. PVC and many other plastics contain high levels of dibutyltin as a stabilizer. Polyethylene adsorbs butyltins. Teflon or polycarbonate are acceptable. Our samples are collected directly in polycarbonate bottles using a sampler designed in our laboratory. Samples can also be collected with a peristaltic pump using viton or silicone tubing at the pump head and teflon transfer lines. The metal parts of a pump head are important in minimizing sample contamination. Pump heads containing brass have

proven to be unsuitable for water sample collection due to very large amounts of tin contamination. Any pumping system used to collect water samples must be completely checked for contamination sources from pump and tubing components by analysis of butyltin free water circulated through the system prior to field use. Three replicate water samples per station and per depth are collected. This permits vertical determination of TBT values in the water column. Samples are collected approximately 0.5 metres below the surface and approximately 0.5 to 1 metres above the bottom sediment. The latter samples are considered samples representative of the bottom water layer.

B. Tissue sampling

1) Three replicate bivalve mollusc samples, each containing five specimens, are collected in the field at each station and stored frozen in ziplock plastic bags if dissection and removal of the soft body parts is not immediately possible.

C. Sediment sampling

1) Sediment samples are collected with a stainless steel grab sampler. One grab per replicate is made with three replicates per station collected. Sediment is collected from the top 2 cm of a grab sample by scooping the sediment into plastic sample bottles. A stainless steel or teflon scoop is used to collect the surface sediment from the grab sample. Enough sediment is collected from each replicate to fill a 250 ml sample bottle by approximately 25-33%. A minimum of 20 grams per replicate is required.

III. SAMPLE CONTAINERS

All water samples are collected in polycarbonate bottles. These containers have been shown to be non-adsorptive for tin compounds (references 1 & 2). The same bottle is used for collection and storage. Sediment samples are stored in 250 ml polyethylene bottles. Dissected tissue samples are stored in 50 ml polycarbonate tubes.

IV. SAMPLE STORAGE

All samples are frozen after collection. Frozen storage of water samples has been shown to be effective for a period of at least three months with respect to tbt (references 3 & 4). A long term evaluation of frozen storage of sediment and tissue samples is currently being done.

V. ANALYTICAL TECHNIQUES

The analytical technique for measuring butyltins in seawater is reported in detail in reference 5. Optimized techniques for measuring butyltins at the sub-part-per-trillion level in seawater and at the part-per-billion level in tissues and sediments are presented in reference 6. An automated system for measuring butyltins in seawater is described in reference 7.

A. Seawater procedure:

Analysis of tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT) in seawater samples is performed by purge and trap/hydride derivatization followed by atomic absorption detection (HDAA). This method requires no preconcentration or extraction and has very low detection limits (0.18-0.36 ng/l). Briefly, a sample is placed in a 500 ml modified gas washing bottle and acidified to pH 5.0-5.5 with 10% acetic acid. Tin hydride derivatives are formed by the addition of 4% sodium borohydride (NaBH_4) prepared in a 1% sodium hydroxide solution in distilled water. A ratio of 1 ml of NaBH_4 to 100 ml of sample is used to generate the hydride species which are subsequently purged from solution and trapped in a glass U-tube. The trap is packed with 20-30 mg of 3% OV-1 on 80/100 mesh Supelcoport and immersed in liquid nitrogen during the purging period. The butyltin species are separated and detected sequentially according to their boiling points as they distill from the trap upon its removal from the liquid nitrogen bath. Detection is accomplished in a quartz burner mounted in an atomic absorption spectrometer using a hydrogen-air flame at 286.3 nm. Complete details of the analytical procedure and the quartz burner design are described in reference 6.

B. Tissue and sediment procedure:

The tissues and sediments are homogenized and extracted with methylene chloride after acidification. Appendix B (reference 11) contains a detailed description of analytical procedures for extracting organotins in soft tissues of marine organisms. The butyltins in the organic layer are derivatized with hexyl magnesium bromide and analyzed by gas chromatography with a flame photometric detector. Improvement in precision and reproducibility in measurement of butyltins in tissues and sediments was attained by adjustment of the concentration in an organic extract to minimize matrix effects, and use of internal standards. This is discussed in detail in Appendix A (reference 6). The absolute detection limits in tissues and sediments are 0.1 ng for TBT, 0.12 ng for DBT and 0.29 ng for MBT.

**MESO STANDARD OPERATING PROCEDURE
ANALYSIS OF ORGANOTIN COMPOUNDS IN
WATER, SEDIMENT, AND TISSUE**

**FEBRUARY 1992
PAGE 4 OF 8**

VI. VERIFICATION OF PROCEDURE

The method has been verified by an inter-laboratory comparison with a National Bureau of Standards (NBS) laboratory (reference 3). Gas chromatography-mass spectrometry (GC/MS) has been used to confirm the presence of tributyltin in an environmental water sample following derivatization to the hydride species (reference 8).

Analysis QA/QC:

Every analytical session:

- Blanks run
- Standard curve run
- NBS QA standard run for seawater samples
- Standards repeated every 6th sample
- Internal standards run with tissues and sediments

Periodically:

- Field spiked samples run
- Standard additions made to environmental samples
- Intercalibration of all instruments and operators

VII. REAGENT AND STANDARD PREPARATION

A. Reagents:

Water: All reagents are prepared in 18 megohm deionized water, unless otherwise noted. Bottled distilled water, available in most grocery stores can also be used if the former is unavailable.

Sodium borohydride solution: 4 g of sodium borohydride (NaBH_4 - "Baker Analyzed 98%") are dissolved in 100 ml of water containing 1 g of sodium hydroxide. This solution is prepared fresh daily and is enough to do 20 runs minus any syringe flushes.

Acid solution: 10 ml of acetic acid are dissolved in 100 ml of 18 megohm deionized water to make a ten percent solution. This solution is prepared fresh weekly or as needed.

Seawater: Seawater for blanks, sample dilution and reaction bottle rinses must be collected from an uncontaminated source. Seawater collected from tidepools removed from heavy boating activity (i.e. bays, marinas or launch sites) or other pristine sources (such as directly from the seawater flume at the Scripps Institution of Oceanography pier in La Jolla, Ca.) must be used.

B. Glass Traps:

Silanized glass traps: Active sites in the trap which bind the hydrides and cause peak reduction or broadening must be avoided. This is done by thoroughly cleaning and silanizing the traps. Variability in sensitivity will be encountered depending on how well the quartz traps have been silanized. A successful method is to clean the traps thoroughly by soaking twice in a critical cleaner such as hot 2% RBS 35, Rinsing with deionized or distilled water and drying. The traps are then washed with hexane and methanol and dried in an oven. While still warm they are filled with Silon CT and allowed to sit for at least 30 minutes. The traps are then thoroughly flushed with hexane and then methanol and conditioned in a 200 C oven for 10-15 min.

Glass traps are 3 mm i.d. glass U-tubes with 2.5-3 inch sides. Hands must be thoroughly clean, oil-free, and dry before attempting to pack silanized glass traps. Hands should be washed with warm soapy water and rinsed with water and alcohol and thoroughly dried. A small pinch of silane treated glass wool is formed into a small ball and pushed into the trap with a clean wood or glass rod. It takes some practice to get a feel for the proper amount of glass wool. If too much is used it seems to result in peak broadening. If not enough glass wool is used the packing material becomes loose, resulting in loss of sensitivity and poor peak definition. A small amount of packing material (20-30 mg of 3% OV-1 on 80/100 Supelcoport) is placed in a folded piece of weighing paper and transferred to the tube. This amount of packing should occupy about 2 cm and is secured with another glass wool plug. Care must be taken not to compress the packing trap too tightly with the second glass wool plug because this causes peak broadening. Broad peaks seem to be mainly caused by two conditions, poorly packed traps and/or moisture accumulation in the system. However, the second plug must be secure enough to prevent the packing material from shifting.

C. Cleaning:

All volumetric glassware and polycarbonate collection bottles are cleaned by soaking in hot 2% RBS 35, rinsed with hot tap water and air dried. Any portion of the system that comes in contact with the tin hydrides is cleaned by sonicating in hot 2% RBS 35, rinsed with distilled water and methanol and dried with helium or other dry gas flow.

D. Standards:

Primary standards: Three 50 ml volumetric flasks are weighed on an analytical balance that is preferably accurate to .01 mg (.00001 g) although .1 mg (.0001 g) accuracy is permissive. This weight is recorded as the tare weight. Each primary standard should ideally contain .1 mg/ml of the butyltin chloride. The mono- and tri-butyltin chlorides (MBT and TBT) are liquids and each can be dispensed into its flask with a 5 μ l Eppendorf pipette. The volumetrics are then weighed again to obtain the gross weight. The tare weight is subtracted from the gross weight to obtain the weight of the butyltin which should be about 5 mg (.005 g). The flasks do not have to contain exactly 5 mg because they will be further diluted to make secondary standards. But the weights must be accurate and good analytical weighing procedures followed. The flasks are then diluted to the mark with ethanol, labeled and stored in the refrigerator. Dibutyltin dichloride (DBT) is crystalline and therefore it is more difficult to transfer approximately 5 mg. A few small crystals are removed from the bottle with the tip of a small spatula and carefully transferred to the tared volumetric flask. Nothing must be spilled or the whole area must be carefully cleaned and another flask tared. The spatula must be immediately cleaned or stored so that it will not cause future contamination problems. The flask is weighed, the gross weight recorded and the tare weight subtracted to give the weight of the dibutyltin dichloride. The flask is diluted to the mark with ethanol and stored as the previous two primary standards. Primary standards are prepared once a month. If undenatured ethanol is unavailable methanol can be used to prepare standards. If methanol is used primary standards should be prepared twice a month.

Secondary (working) standards: A mixed secondary working standard is prepared every other day. The primary standards are allowed to come to room temperature and an appropriate aliquot (100, 50 or 25 μ l) of each is dispensed with an Eppendorf pipette into a 100 ml volumetric flask. This flask is filled to the mark with ethanol (methanol can also be used) and labeled with the date and each concentration.

**MESO STANDARD OPERATING PROCEDURE
ANALYSIS OF ORGANOTIN COMPOUNDS IN
WATER, SEDIMENT, AND TISSUE**

**FEBRUARY 1992
PAGE 7 OF 8**

E. Sample standard preparation data:

Primary standards:

TBT	Gross weight	37.69876 g	
	Tare weight	<u>37.69394 g</u>	
	Net weight	.00482 g	= 4.82 mg/50 ml = .0964 mg/ml
DBT	Gross weight	36.99843 g	
	Tare weight	<u>36.99038 g</u>	
	Net weight	.00805 g	= 8.05 mg/50 ml = .161 mg/ml
MBT	Gross weight	37.47437 g	
	Tare weight	<u>37.46645 g</u>	
	Net weight	.00792 g	= 7.92 mg/50 ml = .158 mg/ml

Secondary standards:

100 µl TBT		= 96.4 ng/ml
50 µl DBT		= 80.5 ng/ml
50 µl MBT	diluted to 100 ml	= 79.2 ng/ml

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MESO STANDARD OPERATING PROCEDURE
ANALYSIS OF ORGANOTIN COMPOUNDS IN
WATER, SEDIMENT, AND TISSUE

FEBRUARY 1992
PAGE 8 OF 8

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Optimization of butyltin measurements for seawater, tissue, and marine sediment samples

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Optimized techniques for measuring butyltins at the sub-part-per-trillion (ppt; $1:10^{12}$) level in seawater and at the part-per-billion (ppb; $1:10^9$) level in tissues and sediments are presented. Purge and trap/hydride derivatization followed by atomic absorption (AA) detection was optimized to give better sensitivity than was previously attained for seawater, yielding environmental detection limits of $0.08\text{--}0.2\text{ ng dm}^{-3}$. Improvement in precision and reproducibility in measurement of butyltins in tissues and sediments was attained by adjustment of the concentration in an organic extract to minimize matrix effects and by use of internal standards. The tissues and sediments were homogenized and extracted with methylene chloride (CH_2Cl_2) after acidification. The butyltins in the organic layer were derivatized with hexylmagnesium bromide and analyzed by gas chromatography (GC) with a flame photometric detector (FPD). The absolute detection limits in tissues and sediments were 0.1 ng for tributyltin (TBT), 0.12 ng for dibutyltin (DBT) and 0.29 ng for monobutyltin (MBT).

Keywords: Tributyltin, dibutyltin, monobutyltin, seawater, tissues, sediments, hydride, Grignard, gas chromatography, atomic absorption, flame photometric detection

INTRODUCTION

Increasing controversy over the use of tributyltin (TBT)-based antifouling paints and their potential impact on the marine environment has necessitated the

development of techniques to measure part-per-trillion (ppt; $1:10^{12}$) levels in seawater and part-per-billion (ppb; $1:10^9$) levels in tissues and sediments. Two recent conferences^{1,2} have produced reference documents on the chemistry, biology, toxicity, fate, distribution and behaviour of organotin compounds in the environment. Monitoring TBT in mussels and oysters is important as studies have shown sublethal effects at sub-part-per-billion levels (Thain in Ref. 1). Because of its lipophilic character, TBT is bioaccumulated by oysters and mussels. Bioconcentration factors from 5000 to 50 000 have been reported.³ The measurement of TBT and its degradation products in the sediment is also relevant because TBT partitions to sediments which may act as a sink and future source of the compound. To evaluate environmental risks, practical, reproducible, and sensitive analytical methods are essential in order to measure and speciate low levels of butyltins in seawater, tissues and sediment.

There are several well-documented methods for TBT analysis in environmental water samples. Reported methods include simultaneous hydride derivatization and extraction⁴ and extraction followed by Grignard derivatization.⁵⁻¹⁰ Direct determination and speciation of tin and alkyltins in seawater can be achieved by hydride derivatization followed by purging and trapping the evolved hydrides. The tin hydrides are then volatilized and detected by hydrogen flame atomic absorption spectroscopy (AA) in a quartz burner.¹¹ Herein we report optimizations of our method,¹² which has increased the sensitivity 10-fold to sub-part-per-trillion levels. Intercalibration using different techniques has given comparable results for butyltin seawater concentrations.¹³

A recent intercalibration study (Stephenson *et al.* in Ref. 2, p. 1334) among seven laboratories illustrated the difficulties of TBT quantitation in tissues and

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sediments. TBT concentrations reported for tissues varied by factors of two to three and most laboratories were not able to participate in the sediment analysis. Our main concern in developing a suitable protocol for organotin analysis of tissues and sediments focused upon two points: precision and sensitivity. We decided to adapt the extraction/Grignard derivatization method^{7-9,14-16} since derivated extracts were chemically and thermally stable. Using this method, TBTCI may be extracted from 5–10 g of tissue or sediment and quantitated without significant matrix effects provided Florisil clean-up, an internal standard, and a correct solvent/sample ratio are used.

EXPERIMENTAL

Seawater analysis

Reagents

Sodium borohydride (NaBH_4 ; 98%) from J.T. Baker was dissolved in 18-M Ω deionized water containing 1% sodium hydroxide AR from Mallinckrodt. The 4% NaBH_4 solution was prepared fresh daily. Baker-analyzed reagent-grade acetic acid was dissolved in 18-M Ω deionized water to make a 10% solution. UPC-grade helium and hydrogen and zero-grade air were used. Mono-, di-, and tri-butyltin chlorides (MBT, DBT, TBT) were used as supplied by Aldrich (Milwaukee, WI). Punctilious ethyl alcohol, (95%) 190 proof USP, was obtained from US Industrial Chemical (Anaheim, CA). Seawater for blanks and sample dilution was taken directly from the seawater flume at the

Scripps Institution of Oceanography pier in La Jolla, CA.

Standards

Primary standards were prepared bimonthly by weighing mono-, di-, and tri-butyltin chlorides into volumetric flasks and diluting with ethanol. Each primary standard contained approximately 100 ng dm^{-3} of the butyltin chloride. The primary standards were stored at 4 °C in the dark. A mixed secondary working standard was prepared every other day by diluting 100 μL of each of the primary standards to 100 cm^3 resulting in a concentration of approximately 0.1 ng μL^{-1} . Standard curves were prepared daily by adding 10, 25, 50 and/or 100 μL of the mixed secondary standards to 500 cm^3 of blank seawater (Fig. 1). A standard was repeated after every sixth sample. Occasional standard additions were made to samples to confirm the absence of matrix effects. Results were calculated as the butyltin cation.

Equipment

Butyltin hydrides were detected with a Buck Scientific model 200 atomic absorption spectrometer fitted with a tin hollow-cathode lamp. This small, relatively inexpensive (< \$8 000) spectrometer has good sensitivity for tin. The absorbance data were recorded on a Shimadzu CR3A Chromatopac recorder as peak areas. The spectrometer and the recorder were plugged into a Microstar energy converter to prevent erratic results from power surges. Cole Parmer 150 mm high-resolution flowmeters were used to control gas flow rates.

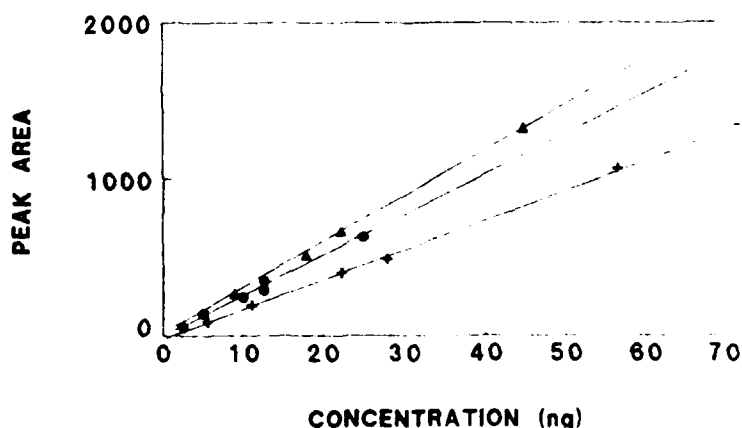


Figure 1 Cation calibration curve for butyltins in seawater. +TBT; Δ DBT; \bullet MBT.

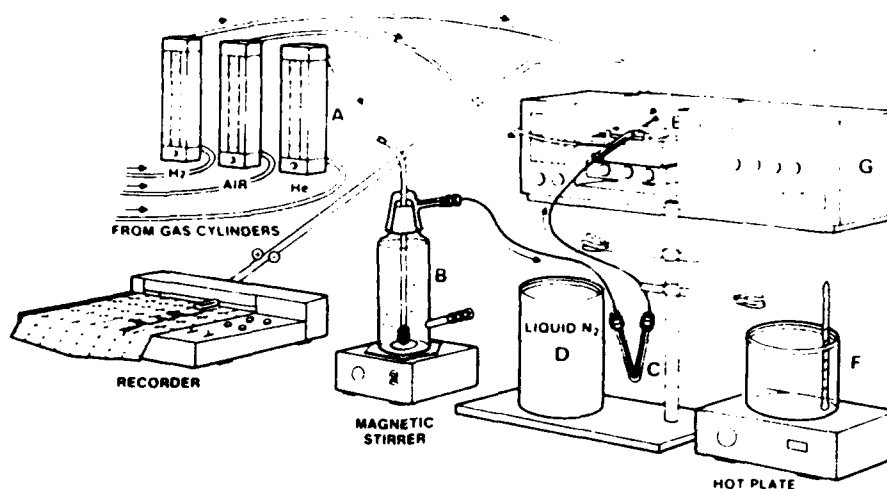


Figure 2 Hydride generation/atomic absorption spectrometry system for measurement of organotin species. A. Helium carrier gas line. B. hydride generator, modified gas washing bottle; C. 3% OV-1 Chromosorb packed glass trap. D. liquid nitrogen for cryogenic cooling. E. quartz burner; F. 180°C high-temperature silicone oil bath; G. Buck atomic absorption spectrometer

Apparatus and optimization

The optimized system (Fig. 2) consists of a modified 500-cm³ gas washing bottle (hydride generator) with an outlet on the top and an injection port on the side. Teflon tape was wrapped around $\frac{1}{8}$ -inch o.d. Teflon tubing to form a secure seal when the tubing was threaded through the top outlet and pulled snug.* This tubing was then pressure-fitted into the end of a 3-mm i.d. glass U-tube (2.5–3-inch sides) which formed the cryogenic trap for the tin hydrides. Avoiding the use of Swagelok fittings which can form dead spaces improved the analysis. The gas washing bottle must also be filled within 1.5 inches from the neck of the bottle to minimize dead space. We have found the tall cylindrical shape of the modified gas washing bottle coupled with the use of a Kimax 12C frit to disperse the helium bubbles provided the most efficient combination to scrub the evolved tin hydrides from the sample. Coarser frits yield larger bubbles that do not remove the hydrides as efficiently. Finer frits gave rise to too much back pressure, thus causing problems in controlling the helium flow. The most effective helium flow rate was found to be 50–60 cm³ min⁻¹. Flow rates below 40 cm³ min⁻¹ did not provide efficient scrubbing and those above 70 cm³ min⁻¹ produced too much water vapor.

Active sites in the trap which bind the hydrides and

cause peak reduction or broadening must be avoided. This was done by thoroughly cleaning and silanizing the traps. We have encountered variability in sensitivity depending on how well the glass traps have been silanized. The method we have found to be most successful was to clean the traps thoroughly by soaking in a critical cleaner such as hot 2% RBS 35 (VWR Scientific, San Francisco Ca.), rinsing with distilled water and drying. The traps were then washed with hexane and methanol and dried in an oven. While still warm they were filled with silanizing fluid (Supelco Silon CT) and allowed to sit for at least 30 min. The traps were then thoroughly flushed with hexane and methanol and conditioned in a 200 °C oven for 10–15 min. Then 20–30 mg of 3% OV-1 on Chromosorb W-HP 80/100 mesh was added to the traps and this packing material was secured with silanized glass wool plugs.

We have also investigated the use of Teflon traps with the same internal diameter as the glass traps. The Teflon traps gave similar sensitivity to silanized glass traps. However, the Teflon did not conduct heat well and water vapor condensed in ends of the trap resulting in greatly reduced sensitivity after a few runs. We believe that an electrothermal heater (Clavell *et al.* in Ref. 1, p. 1152) will make possible use of the Teflon traps.

The trap was connected to a quartz burner with another Teflon tube. It was obvious from the work by

* 1 inch = 2.54 cm

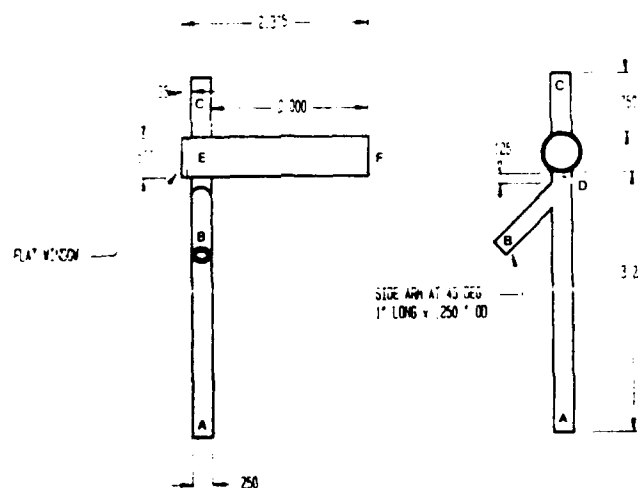


Figure 3 Closed-end quartz burner (dimensions in inches). A, Sample entry; B, air entry; C, hydrogen entry; D, position of glass capillary tip in Teflon tube that introduces tin hydrides into burner; E, hottest portion of burner (500°C); F, open end (400°C)

Donard *et. al.*¹⁷ that the design of the quartz burner could influence sensitivity. We tested their burner design along with several others. We found that the closed-end burner with gas entry off-center gave optimum sensitivity (Fig. 3). External heating of this burner design with a nichrome wire coil did not seem to increase sensitivity. It took the system longer to stabilize after being initially turned on with the heated burner designs. The position of the tube that introduces the tin hydrides influenced sensitivity. A glass capillary (0.5–0.75 inch long) was forced into the end of the Teflon tube from the trap to prevent the end from burning. The tip of the capillary was positioned at the edge of the quartz burner barrel (D in Fig. 3) and fastened into place with a Swagelok reduction fitting at the sample entry (A in Fig. 3). We believe that the increased sensitivity of this burner design results from concentrating the evolved tin hydrides in a small area (E in Fig. 3) resulting in more tin atoms in the lightpath and therefore increased light absorption. This area is also the hottest part of the burner. The open end (F in Fig. 3) of the burner was ignited with a piezoelectric Lab Lyter. It was important to check periodically to ensure that the hydrogen flame was burning. The quartz burner was wrapped with a 2 mm thickness of Fiberfrax ceramic fiber (Lab Safety Supply, Janesville, WI) and mounted on a custom-made adjustable aluminum frame placed on the AA burner head. We have found this ceramic fiber to be a good replacement for poten-

tially carcinogenic asbestos. The burner was then positioned to give maximum light transmission.

Operating procedure

Seawater samples of 10–500 cm³ were introduced into the hydride generator and diluted with blank seawater until the level was within 1.5 inches of the neck of the vessel. The samples were then acidified to approximately pH 5.5 by the addition of 10% acetic acid. The magnetic stirrer was started and helium bubbled through the sample while the glass trap was placed in liquid nitrogen. The trap was immersed just sufficiently to cover the packing material to avoid accumulation of water vapor, and 5 cm³ of 4% sodium borohydride in 1% sodium hydroxide was then injected through the septum and the evolved tin hydrides purged into the cryogenic trap with helium.

After the sample had purged for 5 min, the trap was removed from the liquid nitrogen and the various tin and alkyltin hydrides were detected sequentially, according to their boiling points, as they volatilized from the trap into the quartz burner. The first tin species volatilized from the trap as it came to room temperature was tin hydride (SnH₄). The methyltin hydrides were followed by monobutyltin hydride (BuSnH₃). We have not routinely quantified inorganic tin or methyltins because they were almost always present in small quantities in seawater and generally they were not anthropogenic in origin. After the mono-

butyltin hydride was detected the trap was placed in a water bath at 50 °C to volatilize the dibutyltin hydride (Bu_2SnH_2) as a sharp peak. Volatilization of tributyltin hydride (Bu_3SnH) was achieved by heating the trap in a 180 °C silicone oil bath. The butyltin hydrides were atomized in a hydrogen/air flame using the quartz burner discussed earlier. The hydrogen and air flow rates were 220 and 140 $\text{cm}^3 \text{min}^{-1}$ respectively. The tin species were detected by atomic absorption spectroscopy at a wavelength of 286.3 nm and a tin hollow-cathode lamp current of 8 mA. Use of the 224.6 nm wavelength gave somewhat better sensitivity but resulted in considerably more baseline noise. It was very difficult to resolve small peak areas with the greater baseline noise so we found that although we had to sacrifice some sensitivity, use of the 286.3 nm wavelength gave better results.

All lines and the trap were periodically flushed with dry helium to remove water vapor. This was important to retain sensitivity and increase the life of the trap. The traps must be thoroughly dried by helium purging between runs. Although we have run at the rate of four samples per hour, we have found more consistent results were obtained at three samples per hour.

Tissue and sediment analysis

Reagents

Mono-, di-, and tri-butyltin chlorides (MBTCl, DBTCl, TBTCl) and 2.0 mol dm^{-3} n-hexylmagnesium bromide were obtained from Aldrich. n-Hexane, glass-distilled dichloromethane, ACS-certified methanol, and Ultrex hydrochloric acid were obtained from VWR.

Standards

TBTCl, DBTCl, and MBTCl stock solutions were prepared bimonthly by weighing mono-, di-, and tri-butyltin chlorides into volumetric flasks and diluting with hexane.

Triptyltin bromide (TPTBr), the internal standard, was prepared by bromination of tetrapentyltin which was prepared from pentylmagnesium bromide and tin tetrachloride.^{18,19}

Equipment

Instrument conditions were as follows. The Hewlett-Packard 5890 gas chromatograph was equipped with a flame photometric detector. The filter, obtained from

Dietrich Optical, had a range of 625–2000 nm. Flow rates through the detector were 100 $\text{cm}^3 \text{min}^{-1}$ for air and 170 $\text{cm}^3 \text{min}^{-1}$ for hydrogen. Nitrogen make-up gas plus helium carrier gas had a combined flow rate of 35 $\text{cm}^3 \text{min}^{-1}$. The column head pressure was 28 psi (193 kPa). The injector and detector temperatures were 225 °C and 250 °C, respectively. The oven program was set as follows: after an initial temperature of 50 °C for 2.00 min, the oven was ramped at 30 °C min^{-1} to a final temperature of 200 °C and held for 8.00 min. A bake-out at 240 °C for 6.00 min was then programmed. The column was a Supelco SPB-1 fused-silica capillary 30 m long and with a 0.25 mm i.d.

Sample preparation

All glassware for preparation and storage was soaked overnight in RBS cleaning solution, rinsed with tap water, and soaked overnight in dilute nitric acid. Sediments and tissues were stored frozen in polycarbonate tubes. Extractions were carried out in disposable polypropylene centrifuge tubes. A sample set typically consisted of 12 samples, a blank, and a standard addition set.

Tissue Excess of water was decanted from the tissues. Approximately 5 g of wet tissue were homogenized using a Tekmar tissuemizer. This tissue was weighed to the nearest 0.1 g in tared 50- cm^3 polypropylene centrifuge tubes, and 10 cm^3 of 1.5 mol dm^{-3} HCl and 20.0 cm^3 of methylene chloride were added to each tube. The capped tubes were mixed on a vortex mixer for 2 min and placed on a rotary mixer for 3 h. After centrifugation, a 2.0- cm^3 aliquot was removed from the bottom layer with a Pasteur pipet and dried under air in a 35 °C water bath.

The extracts were redissolved in 2 cm^3 of hexane and the internal standard, triptyltin bromide, was added. A second internal standard, tripropyltin chloride, was sometimes added before extraction of the tissue. n-Hexylmagnesium bromide (250 μL of 2.0 mol dm^{-3}) was added to each extract. After 10 min, 2 cm^3 of 0.2 mol dm^{-3} sulfuric acid were added to hydrolyze the remaining Grignard reagent. The top layer was removed and eluted on Supelco florasil columns (and Supelco manifold) with hexane. The extracts were dried under air as before and reconstituted in 200 μL of hexane.

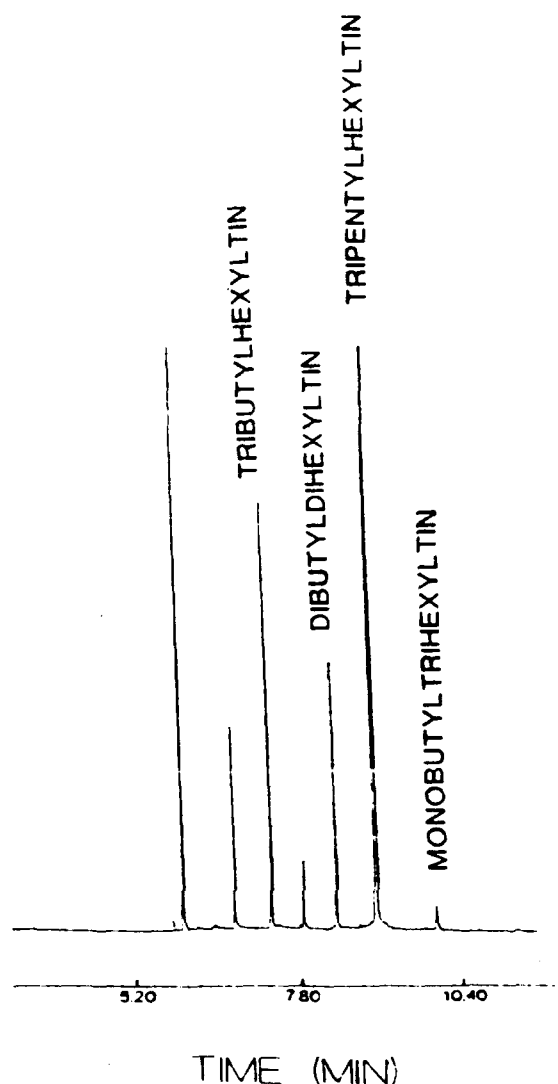


Figure 4 A typical environmental chromatogram from a moderately TBT-impacted San Diego Bay sediment.

Sediment After excess water had been decanted and the sediment had been air-dried overnight, approximately 5 g of the partially dried sediment were placed into a tared polypropylene centrifuge tube, ground with

Table 1 Calibration data

Compound	N	Average $r_f \pm s$	Limit of detection (ng)
TBT	5	1.39 ± 0.17	0.10
DBT	5	1.19 ± 0.21	0.12
MBT	4	0.48 ± 0.14	0.29

All peak areas are normalized using the internal standard. TPT, N represents the number of calibration standards used to determine the average response factor (r_f) values. The concentration range for TBT is 0.148–1.288 $\mu\text{g cm}^{-3}$.

Table 2 TBT spiked recoveries of mussels and sediments from San Diego Bay

Measured ($\mu\text{g cm}^{-3}$)	Actual ($\mu\text{g cm}^{-3}$)	Recovery (%)
(A) Mussels		
0.13	0.12	108
0.24	0.29	83
1.07	1.18	91
3.21	3.15	103
(B) Sediments		
0.30	0.23	130
0.57	0.54	105
1.09	1.01	93
1.83	1.80	102

a pestle to break up any clumps, and weighed to the nearest 0.1 g; 10 cm^3 of 1.5 mol dm^{-3} HCl in methanol and 20.0 cm^3 of methylene chloride were added to each tube. The capped tubes were mixed on a vortex mixer for 2 min. After venting gases, the tubes were placed on a rotary mixer for 3 h. Sediment preparation was completed in the same manner as tissues. Figure 4 shows typical results.

Calibration

Calibration for tissues and sediments as seen in Table 1 was based upon the response factors of four calibration standards in the concentration range 0.125–1.25 $\mu\text{g cm}^{-3}$ where the response factor (rf) was calculated as follows:

$$rf = \frac{\text{X peak area}}{\text{internal standard peak area}} \times \frac{\text{concentration of internal standard}}{\text{concentration of X}}$$

Calibration standards were processed and quantified every two weeks to check analytical control (using linear regression analysis and rf) as well as the condition of the instrument. Generally the rf will not deviate by $\pm 20\%$ throughout the life of the column. The butyltin peak areas are normalized against the internal standard, triphenylhexyltin.

Spiked recoveries

Table 2 illustrates spiked recovery data for mussels and sediment from San Diego Bay. Diluted TBTCI stock solution was added in linear volumetric amounts to four 10 g aliquots of homogenized tissue and sediment. One blank aliquot of tissue and sediment was processed as well to check if recoveries were similar for different spike concentrations (i.e. by linear regression). The spiked samples were refrigerated overnight before processing.

Optimization of methylene chloride extraction/Grignard derivatization for solids analysis

There were several advantages in using the methylene chloride extraction/Grignard derivatization method. Methylene chloride is a semipolar solvent that extracted TBT with greater than 90% recovery. DBT-spiked recovery from sediment was better than 90% but MBT had less than 12% recovery and the standard deviation was greater than $\pm 100\%$. A complexing agent such as tropolone would increase the extraction efficiency of other alkyltins but did not increase the recovery of TBT and caused non-linear recoveries in standard addition tests. More importantly, the tetra-alkyltin derivatives were thermally stable in the GC injector. Our experience and studies using a GC MS have shown that anion exchange occurs for both alkyltin halides and alkyltin hydrides,²⁰ dependent upon the history of the injector. In addition, the tetra-alkyltin derivatives were chemically stable over a 10-day period (if frozen), and were amenable to Florisil

clean-up which removes some of the more polar compounds as well as particulates.

The disadvantages of using this method are that signal damping may occur due to co-elutants in the detector flame, and that evaporative losses of TBTCI may be significant at high alkyltin concentrations. In particular, we noticed a signal damping of the internal standard relative to other peaks that resulted in higher quantitated butyltin concentrations. Given a solvent/sample ratio of 20 cm³ 5 g, we also noticed that concentrating the extracts by 20-fold or more caused an erratic internal standard recovery. Since spiked recoveries of the internal standard from sample blanks were better than 90%, the apparent lower recoveries might have been an artifact caused by co-eluting compounds in the detector flame.²¹ Concentrating the matrix one- or two-fold reduced this effect and sensitivity of the method was not significantly compromised. Usually a comparison of a linear regression analysis of standard additions against that of calibration standards indicated whether significant matrix effects were present. Studies by Craig *et al.*²⁰ have shown that significant evaporative losses of TBT⁺ occurred at concentrations greater than 3000 ng g⁻¹. To avoid this, we reduced the concentration and drying time needed by drying and derivatizing a 2-cm³ aliquot of the methylene chloride layer. Extracts were removed before complete dryness. Standard deviations for spiked recoveries and ambient environmental samples were normally 20% or less and average spiked recoveries were greater than 95% (Tables 2 and 3).

RESULTS AND DISCUSSION

Figure 5 shows typical results of the analysis of a seawater sample with fairly low butyltin concentrations. Calibration standards are linear to at least

Table 3 Data from San Diego Bay mussels ($\mu\text{g g}^{-1}$ wet weight)

Station	MBT (\pm SD)	DBT (\pm SD)	TBT (SD)
SDM4 19	0.076 (\pm 0.004)	0.169 (\pm 0.007)	0.252 (\pm 0.020)
SDM4 53B	0.257 (\pm 0.029)	0.538 (\pm 0.025)	1.067 (\pm 0.220)
SDM4 2B	BDL \pm ^a	0.087 (\pm 0.016)	0.068 (\pm 0.011)

Three replicates are analyzed for each station. Each replicate consists of approximately five homogenized mussels for a total of 15 mussels per station.

^aBelow detection limit.

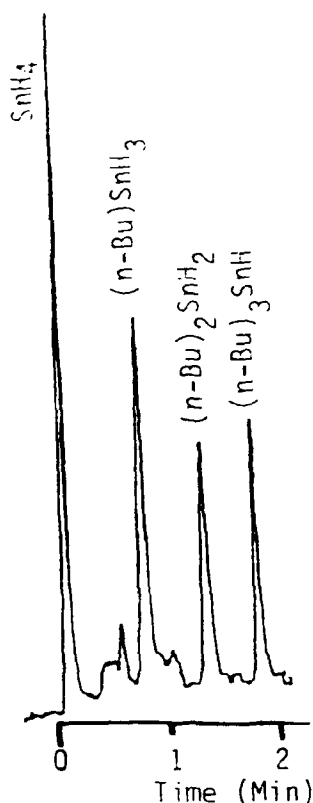


Figure 5 Speciation of butyltins in seawater. Quantities of the compounds as the cation are as follows: $(n\text{Bu})\text{Sn}^{3+}$, 1.3 ng; $(n\text{Bu})_2\text{Sn}^{2+}$, 64 ng; $(n\text{Bu})_3\text{Sn}^{+}$, 1.3 ng

70 ng dm^{-3} as shown in Fig. 1. Seawater samples from marinas where high butyltin concentrations were expected were diluted with blank seawater to keep the values on the calibration curve, rather than exceeding the limits of linearity.

Our current absolute detection limits were obtained by running a series of blank seawater samples spiked with about 0.5 ng of each butyltin compound. The detection limits based on three standard deviations (σ) of these near-background standard additions were 100 pg for tributyltin, 40 pg for dibutyltin, and 65 pg for monobutyltin calculated as the cation. This resulted in environmental detection limits in seawater of $0.08\text{--}0.2 \text{ ng dm}^{-3}$ as the cation assuming a sample volume of 500 cm^3 . This compares well with the previous lowest TBT detection limit of 1.2 ng dm^{-3} as the cation assuming a sample volume of 100 cm^3 .^{22,23} Our absolute tributyltin sensitivity

ranged from 180 pg to 80 pg. This range reflects differences in the five systems we use for monitoring butyltin concentrations.

Table 3 reports *Mytilus edulis* tissue monitoring data from San Diego Bay. Station SDM4 19 is located near a commercial marine dry dock facility. Average TBT water concentrations were 20 ng dm^{-3} (Seligman *et al.* in Ref. 1). Station SDM4 2B is located at the mouth of San Diego Bay where TBT concentrations were 3 ng dm^{-3} (Seligman *et al.* in Ref. 1). Station SDM4 53B is located in a small yacht harbor where TBT water concentrations are $50\text{--}100 \text{ ng dm}^{-3}$. Similar monitoring studies by Huggett *et al.*¹⁴ have shown TBT concentrations as high as $1.5 \mu\text{g g}^{-1}$ wet weight in oysters taken near a marina. Mussels taken from Sarah Creek (between two marinas and a boatyard) had a mean value of $0.834 \mu\text{g g}^{-1}$ wet weight.¹⁴ A comparison of the detection limits of some analytical methods (Table 4) along with a California Fish and Game intercalibration study (Stephenson *et al.*, Ref. 2, p. 1334) indicates that several methods might be useful for monitoring TBT in tissue and sediment provided care is taken to minimize matrix effects and thus quantitation errors. Recovery data for these methods (tissues) are as follows: 100% (Ref. 24), 105% (Ref. 25), 96% (our study), 93% (Ref. 26). As mentioned previously, standard additions before extraction (to check procedural variability) or after extraction (to check matrix effects) serve as a good indicator of analytical control.

Table 4 Comparison of some analytical methods

Method	Limits of detection (ng g^{-1} wet weight)	Ref
Hydride generation/AA	11–25	24
$\text{CH}_2\text{Cl}_2/\text{AA}$	2.5	25
$\text{CH}_2\text{Cl}_2/\text{Grignard}/\text{FPD}$	10–20	Present study
$\text{CH}_2\text{Cl}_2/\text{hydride}/\text{FPD}$	10	26

CONCLUSION

In view of recent legislation in California and other states banning the use of TBT antifouling paints on pleasure craft, the ability to measure low levels of TBT will be important in order to monitor compliance and the effects of the ban. By changing burner design and removing active sites from the trap, we have attained

sub-part-per-trillion detection limits of butyltins in seawater. We hope to adapt this hydride method to measure and speciate other organometallic toxicants such as lead, arsenic and selenium in the marine environment.

Improvement in the measurement of butyltins in tissues and sediments was achieved by optimizing extraction parameters and reducing matrix effects. Use of internal standards and quality control checks have also improved our analytical variability.

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Analytical Procedures for Extractable Organotins in Soft Tissues of Marine Organisms

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CONTENTS

1. INTRODUCTION	1
2. OUTLINE AND SUMMARY OF ANALYTICAL PROCEDURES FOR TISSUES	2
3. METHODS FOR TISSUE PREPARATION	5
3.1 Tissue extraction, Day 1	5
3.2 Tissue extract reconstitution, Day 2	8
3.3 Extract preparation for GF-AAS Analysis	9
3.4 Extract preparation for GC-FID and GC-MS Analysis	13
4. SUMMARY OF INSTRUMENTATION	16
4.1 GF-AAS	16
4.2 GC-FID	17
4.3 GC-MS	17
5. SAMPLE CALCULATIONS	19
6. CONCLUDING REMARKS	22

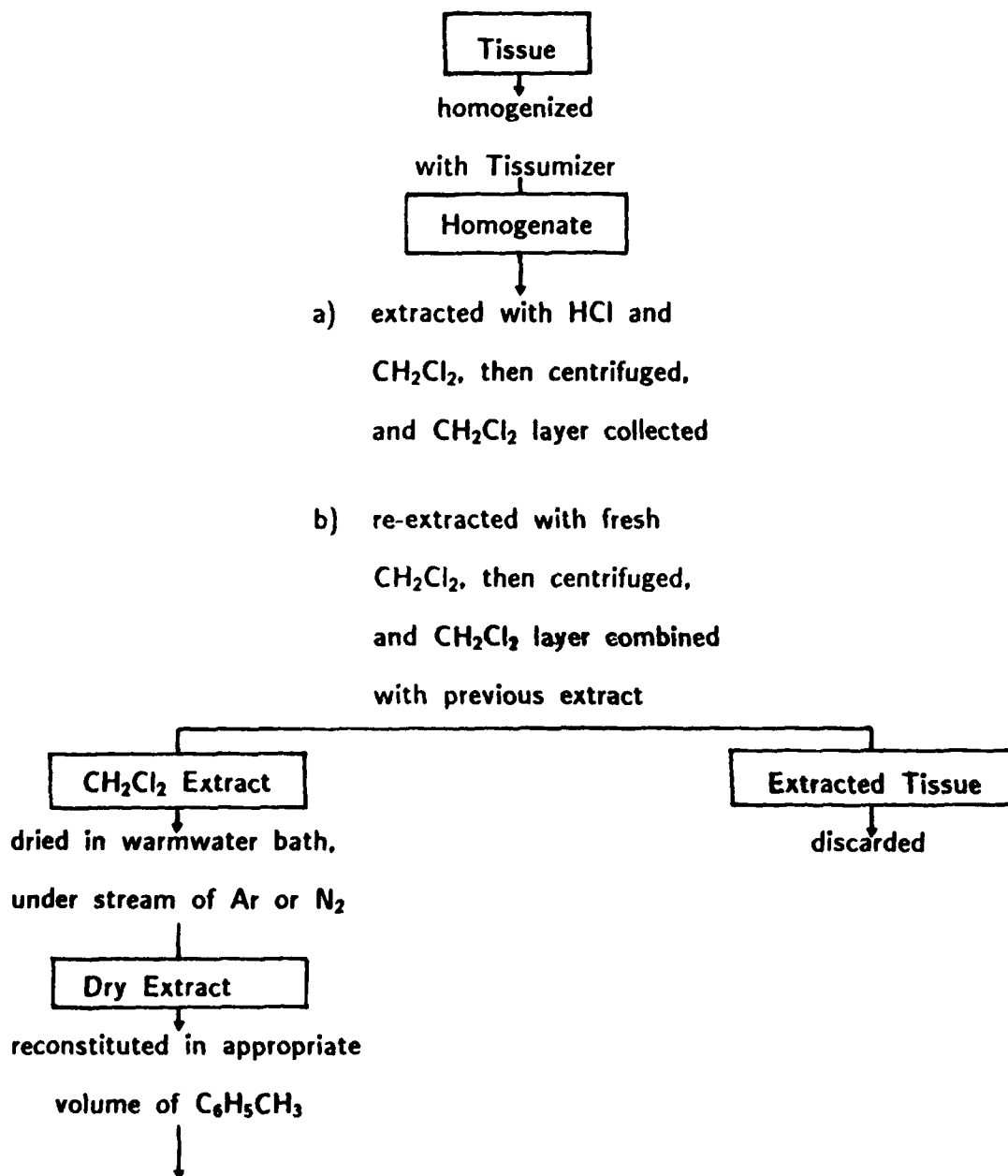
1. INTRODUCTION

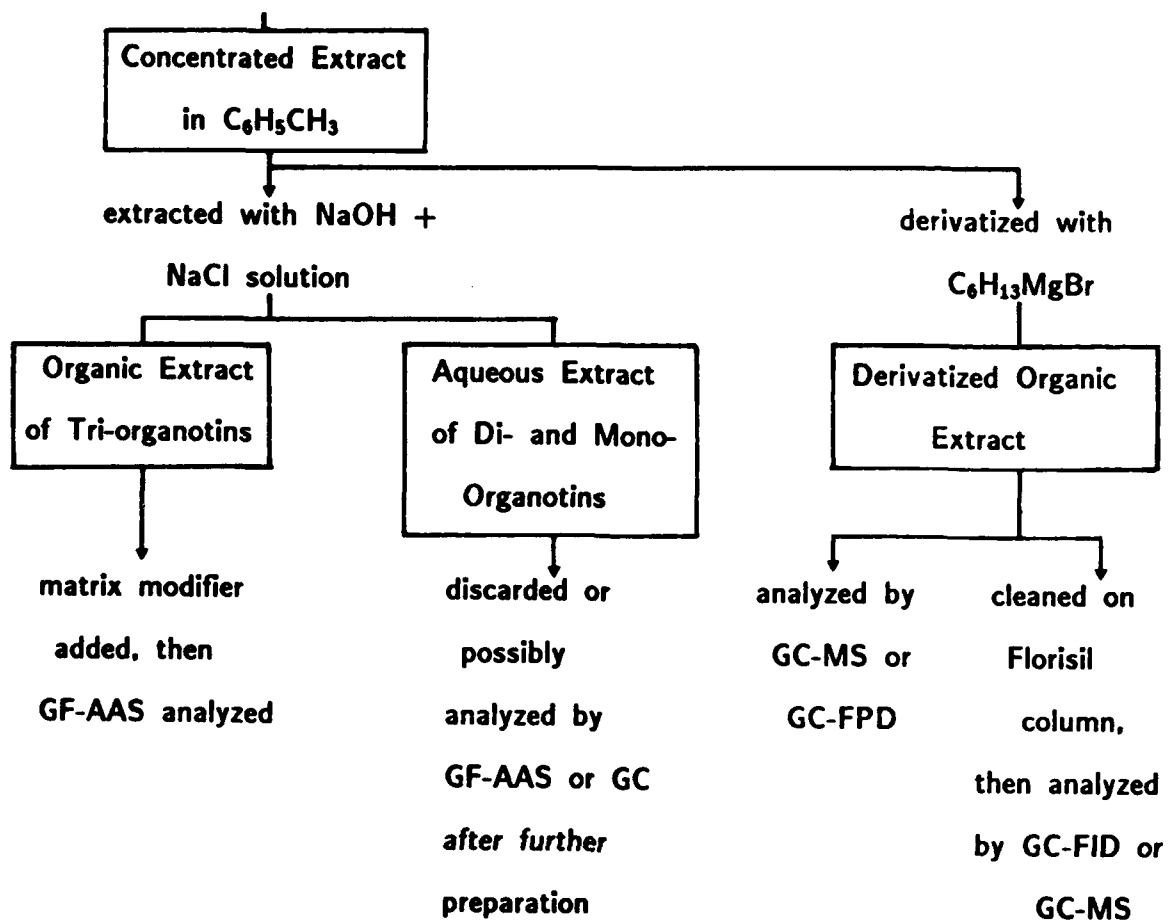
There has been increasing national and international concern about the effects of tributyltins on the marine environment. Tributyltins (TBT) are introduced into the waterways predominantly from their use as antifouling agents in marine paints. These coatings, which release the biocide into the water column, are extensively used on commercial and recreational vessels and are proposed for use on naval ships in the near future.

Due to the toxic nature of TBT, there is an extreme need to assess its impact and fate in relation to non-target organisms. To evaluate the interactive roles of TBT and marine organisms, it is necessary to effectively isolate, identify, and quantify TBT and, at times, its alleged degradation products, di- and monobutyltins (DBT and MBT). The analytical procedures presented here are those currently used at the Ocean Sciences Laboratory, Naval Ocean Systems Center, San Diego, to quantify either (a) extractable TBT only, or (b) both TBT and DBT in various marine organisms. Because it is doubtful that any one method will be effective in all situations, development (i.e., improvement) of the analytical procedures is an ongoing process. For this reason, it is stressed that the procedures presented here are (at the time of this writing) most effective for the samples encountered in this laboratory. The thorough (almost cookbook style) presentation is meant to inform the reader of the complete methods currently in use at NOSC and is by no means intended to be an analytical code or rule.

2. OUTLINE AND SUMMARY OF ANALYTICAL PROCEDURES FOR TISSUES

In general, analytical procedures for butyltins in marine organisms follow the outline below.





The outline of analytical procedures can be summarized as follows. Thaw the frozen sample, (and if not previously shucked, remove tissue from the shell or carapace, pouring off excess fluid), then grind the tissue. Weigh homogenate (5-17 g), add 6 M aqueous hydrochloric acid (HCl), and extract two times with methylene chloride (CH_2Cl_2). Dry the combined extracts in a warmwater bath under a stream of argon (Ar) or nitrogen (N_2).

Reconstitute the residue in an appropriate amount of toluene ($\text{C}_6\text{H}_5\text{CH}_3$). This concentrated extract can be prepared for either graphite furnace-atomic absorption spectrophotometry (GF-AAS), analyzing for tributyltin only, or gas chromatography (GC), analyzing for speciated butyltins.

For GF-AAS analysis, mix the extract with a solution of ~5 M NaCl in 3-percent aqueous NaOH. Discard the aqueous layer or save it for possible further analyses of your choice. To aliquots of the toluene ($\text{C}_6\text{H}_5\text{CH}_3$) layer, make standard additions by adding appropriate volumes of a freshly prepared tributyltin chloride standard, then dilute the solutions with an ammonium dichromate [$(\text{NH}_4)_2\text{Cr}_2\text{O}_7$] and isopropyl alcohol [$(\text{CH}_3)_2\text{CHOH}$] matrix modifier, and analyze the sample by GF-AAS.

For GC analysis, add an internal standard to the toluene concentrate and derivatize with hexylmagnesium bromide ($\text{C}_6\text{H}_{13}\text{MgBr}$). This hexylated concentrate can be analyzed directly by GC-mass spectrometry (GC-MS) or be cleaned by passage through a Florisil column before either GC-flame ionization detection (GC-FID) analysis or GC-flame photometric detection (GC-FPD) analysis.

3. METHODS OF TISSUE PREPARATION

3.1 TISSUE EXTRACTION, DAY 1

Equipment

Glassware and Plasticware (per sample)

- 101- X 38-mm round-bottom polycarbonate centrifuge tube with screw cap (NALGENE)*
- 200- X 25-mm culture tube with Teflon-lined screwcap (KIMAX)
- 127- X 28-mm round-bottom SORVALL centrifuge tube (PYREX), two per sample
- 125-ml Erlenmeyer flask

Chemical Solvents and Reagents

- Water for cleanup, ~300 ml for one to three samples
- Ethanol (C_2H_5OH) for cleanup, ~150 ml for one to three samples
- 6 M hydrochloric acid (concentrated ULTREX HCl, diluted with distilled water), equal weight as sample
- Methylene chloride (CH_2Cl_2), 50 ml per sample

Other Materials

- Dissecting tray*
- Shucking knife*
- Vernier caliper*
- Kimwipes
- Lab marker
- 400-ml plastic disposable beaker, one per two samples
- Rack for 200- X 25-mm culture tubes and 127- X 28-mm round-bottom centrifuge tubes
- 500-ml wash bottle for ethanol
- Tissumizer (TEKMAR)
- Top-loading balance (accurate to 0.01 g)
- Beaker for taring, ~400 ml
- 400-ml plastic disposable beaker for water wash
- ~4- X 140-cm (100-ml) glass graduated cylinder for ethanol wash
- Teflon-coated spatula
- 100-ml glass graduated cylinder (in addition to the one listed above)
- 20-ml glass graduated cylinder
- 1-in.-wide Teflon tape, ~4-in. length per sample
- Parafilm sheeting, four squares per sample
- Polyethylene gloves
- Rotatorque (COLE-PARMER)
- Compressed argon or nitrogen gas
- Mini-vap (SUPELCO), one outlet per sample

*Necessary if animals have not been dissected.

~5- X 30- X 19-cm tray, one per six samples
5- to 10-ml pipettor
Disposable tips for 5- to 10-ml pipettor, one per sample

Procedure

- (1) With a lab marker, label 200- X 25-mm culture tubes (one per sample) and 127- X 28-mm round-bottom SORVALL centrifuge tubes (two per sample), and place them in a rack.
- (2) Cut 1- to 1.5-in. lengths of 1-in.-wide Teflon tape (four per sample), and keep them clean on a Kimwipe.
- (3) Cut squares of Parafilm sheeting (four per sample); keep them clean on a Kimwipe.

For animals not previously shucked into 101- X 38-mm polycarbonate centrifuge tubes, continue with step 4; otherwise proceed with step 14.

- (4) Thaw frozen specimens in their storage bags at room temperature.
- (5) Label 101- X 38-mm polycarbonate centrifuge tubes (three replicate tubes per sample bag) with labeling tape.
- (6) Pool three groups of five to ten animals each (of 45-60 mm length if mussels) and place them on a dissecting tray.
- (7) Using a vernier caliper, measure and record the length of each animal in each set to an accuracy of 0.01 mm (optional).
- (8) Open the shell with a shucking knife and record the reproductive stage of each animal (optional).
- (9) Blot the interior with a fresh Kimwipe.
- (10) (Discard the byssal threads of mussels.) Cut away all tissue from the inner shells.
- (11) Carefully transfer the tissue of all animals in each set into their correctly labeled polycarbonate centrifuge tubes and cap the tubes.
- (12) With water and then ethanol, thoroughly rinse the tray and knife, letting them air-dry before being used for the next sample batch.
- (13) Clean the Tissumizer by immersing the rod in a 400-ml beaker of water and switching the controller "ON" for 5 s. Wipe the rod with a Kimwipe, then immerse the rod into a 4- X 140-cm (100-ml) graduated cylinder of ethanol, and again switch the controller on for ~5 s. Be sure to keep at least the bottom inch of the Tissumizer rod submerged while it is running. Let the Tissumizer air-dry before using it for tissue homogenization.

- (14) (For tissue samples that were frozen in the polycarbonate centrifuge tubes, thaw the samples at room temperature before proceeding.) For each polycarbonate tube of tissue, grind the sample until it appears homogeneously smooth (should take from 1 to a few minutes). Try not to let the sample heat up during this process.
- (15) Wipe the Tissumizer with a Kimwipe and clean the rod as in step 10. Replace dirty water and ethanol with clean batches, at least after each three uses.
- (16) Set the 400-ml taring beaker on the top-loading balance. Into the beaker, place the correctly labeled 200- X 25-mm culture tube. Tare these containers.
- (17) With a Teflon-coated spatula, carefully transfer the tissue homogenate to the tared culture tube.
- (18) With a Kimwipe, wipe the top and outside of the culture tube's lip.
- (19) Record the weight.
- (20) Seal the tube with a piece of Parafilm and place the tube in a rack.
- (21) To each of the culture tubes with the weighed homogenates, add (a) an equal weight of 6 M hydrochloric acid (i.e., more easily measured as 1 ml acid/1 g tissue), and (b) 25 ml of methylene chloride.
- (22) Cross two pre-cut pieces of Teflon tape over the mouth of each tube before capping tightly with a lid and shake each tube vigorously by hand for 30 s.
- (23) Securely place all culture tubes on the Rotatorque and set the controller to ~6.5 on high speed.
- (24) After 1 hr, move the tubes from the Rotatorque to a tube rack.
- (25) For each sample, vigorously shake the culture tube and evenly divide the slurry between the two corresponding 127- X 28-mm round-bottom centrifuge tubes. (A two-pan balance can be used to verify even-weightedness.)
- (26) To each emptied culture tube, add 25 ml of fresh methylene chloride, recap each tube with the Teflon-tape-lined lid, and shake the tubes for ~15 s.
- (27) Cover the mouths of the centrifuge tubes with Parafilm and place the tubes in the centrifuge.
- (28) Set the centrifuge at 3000 rpm for 5 min (start at speed setting 3.5 for ~1 min, then set the speed to 5 for the remaining time).
- (29) When the centrifuge has completely stopped, move the tubes to the tube rack, pairing tubes with their partners.
- (30) Transfer the tape labels from the empty polycarbonate centrifuge tubes to 125-ml Erlenmeyer flasks.

- (31) With a 5- to 10-ml pipettor, transfer the bottom (methylene chloride) layers of the first two matched centrifuge tubes to the appropriately labeled Erlenmeyer flask and place the used pipet tip in the flask.
- (32) Put a fresh tip on the pipettor and proceed to transfer the remaining extracts in a similar manner, using a fresh pipet tip for each sample and being sure to transfer both fractions of each sample to the appropriate flask.
- (33) From each culture tube, evenly divide the methylene chloride rinse between the two corresponding centrifuge tubes (which contain the 1X extracted HCl/tissue mixture).
- (34) Cover the mouth of each centrifuge tube with a piece of Teflon tape.
- (35) With a polyethylene glove on your hand, securely stopper the taped mouth with your thumb and vigorously shake each tube for 30 s. (Change the glove when it becomes soiled.)
- (36) Place the centrifuge tubes in the centrifuge and centrifuge the samples as in step 28.
- (37) Using the 5- to 10-ml pipettor and the tips from the Erlenmeyer flasks, carefully transfer the corresponding methylene chloride extracts as described in step 31. Be sure not to cross-contaminate samples by mixing up tips. (If this is a concern, always use fresh tips.) Discard all soiled tips.
- (38) Place the Erlenmeyer flasks in an approximately 5- X 30- X 19-cm tray (six flasks per tray) and fill the tray halfway with warm (~45°C) tap water.
- (39) Into each Erlenmeyer flask's mouth, position a mini-vap outlet and set the argon or nitrogen gas pressure for a slow, steady stream.
- (40) Every 15-20 min, carefully scoop out and discard the cooled water from the pan, being sure not to tip the flasks, and refill the pan with warm tap water.
- (41) When each extract reaches dryness, secure a square of Parafilm over the mouth of each Erlenmeyer flask.
- (42) Store the flasks in the refrigerator overnight.

3.2 TISSUE EXTRACT RECONSTITUTION, DAY 2

Equipment

Glassware

20-ml borosilicate scintillation vials with polyethylene-lined caps, one per sample

Chemical Solvents

Toluene ($C_6H_5CH_3$), ~5 ml per sample

Other Materials

1000- μ l pipettor
Disposable tips for 1000- μ l pipettor

Procedure

- (1) Bring the refrigerated Erlenmeyer flasks that contain the dried extracts to room temperature.
- (2) Label 20-ml borosilicate scintillation vials, one per sample.
- (3) With a 1000- μ l pipettor, add 3 ml toluene to each flask. (If you have an expectation that the TBT concentration will be $>2 \mu\text{g/g}$ wet tissue, add 5 ml toluene.)
- (4) Swirl and roll each flask so that the toluene rinses the entire inner surface of the flask, being careful not to spill any of the rinse, and transfer the reconstituted extract to the appropriate scintillation vial.

This extract can now be prepared for GF-AAS and/or GC analysis as described in sections 3.3 and 3.4, respectively.

3.3 EXTRACT PREPARATION FOR GF-AAS ANALYSIS

Equipment

Graphite Furnace-Atomic Absorption Spectrophotometer
(see section 4.1 for associated equipment and setup parameters)

Glassware and Plasticware

16- X 100-mm PYREX culture tubes, two per sample
Polypropylene screw caps for culture tubes
Small, ~6.5 cm top diameter, disposable polystyrene funnels, one per sample

Chemical Solvents and Reagents

3-percent aqueous sodium hydroxide solution (NaOH in distilled water)
stored over a layer of methylene chloride (CH_2Cl_2), ~2 ml per sample

Sodium chloride (NaCl), ~0.5 g per sample

Standard of tributyltin chloride [$(C_4H_9)_3\text{SnCl}$] in toluene, ~1-1.5 $\mu\text{g/ml}$

Toluene ($C_6H_5CH_3$), ~5 ml per sample

Isopropyl alcohol $[(\text{CH}_3)_2\text{CHOH}]$, ~5 ml per sample

0.1 M ammonium dichromate $[(\text{NH}_4)_2\text{Cr}_2\text{O}_7]$, ~1 ml per sample

Other Materials

Polyethylene gloves

Rack for 16- X 100-mm culture tubes

Teflon-coated spatula

5-, 10-, 20-, 50-, 100-, 500-, and 1000- μl pipettors and tips

Vortex mixer (VWR)

Polyethylene AA cups, ~5-10 per sample

AA cup rack

Procedure for GF-AAS Analysis of TBT*

- (1) To prepare the primary tributyltin chloride (TBTCl) standard (1° STD), begin by weighing (to 0.0001 g) a 100-ml volumetric flask.
- (2) Add a portion of a drop of TBTCl from a 10- μl pipettor tip to the flask.
- (3) Reweigh the flask.
- (4) Carefully add toluene to bring the standard to the 100-ml volume mark, cap the flask, and shake it well (polyethylene gloves should be worn).
- (5) Calculate the concentration ($\mu\text{g}/\text{ml}$) of TBTCl in the volumetric flask.¹
- (6) If the 1° STD is more concentrated than ~1.5 μg TBTCl/ml, a secondary standard (2° STD) must be made.² Shake the 1° STD, and with a pipettor(s) transfer an appropriate amount of 1° STD to a clean volumetric flask. Bring this 2° STD to volume by carefully adding toluene, then cap and shake the flask.
- (7) Label 16- X 100-mm PYREX culture tubes (two per sample plus two for the TBTCl standard, putting one set aside) and place them in a rack.
- (8) To the first set of culture tubes, add ~0.5 g (two scoops with a Teflon-coated spatula) sodium chloride through a disposable funnel.

*Superscript number after a procedural step refers to corresponding step number in the sample calculation, section 5.

- (9) With a 1000- μ l pipettor, add 2 ml of 3-percent sodium hydroxide solution to each tube intended for the samples, and 3-4 ml to the tube for the STD, being careful not to pick up any of the methylene chloride layer beneath the sodium hydroxide.
- (10) Into each sample in the scintillation vials, pump a 1000- μ l pipettor to mix the extract, transfer 2 ml of extract to the appropriate culture tube, cap the tube tightly, and place the tube on a vortex mixer for 30 s. Be sure to use a fresh pipet tip for each sample, and try not to touch the tip to the interior of the culture tube between transfers.
- (11) For the TBTCI STD (\sim 1-1.5 μ g TBTCI/ml), shake the STD and pipet 3-4 ml to its labeled culture tube, cap, and mix as described in step 10.
- (12) After the two layers in each tube separate completely (5 min or more; some tubes may require light tapping to release bubbles of the organic layer from the salt), pipet most of the top layer to the empty corresponding tube of the second set. Again, be sure to use a fresh pipet tip for each sample. This aliquot of extract will be analyzed by GF-AAS.

Before analyzing the tissue extracts, a standard curve (absorbance vs. concentration) should be produced of the sodium hydroxide-treated TBTCI STD to assess the working status of the GF-AAS. To do this:

- (13) Set up five AA cups within the AA cup rack.
- (14) Add the TBTCI standard and matrix modifiers by following the outline below, using the appropriate pipettors and being sure to change tips after each use to avoid contamination.

Cup No.	μ l of STD	μ l of Toluene	μ l of Isopropyl Alcohol	μ l of 0.1 M Ammonium Dichromate
0	0	300	600	100
1	5	300	600	100
2	10	300	600	100
3	25	300	600	100
4	50	300	600	100

- (15) With a 1000- μ l pipettor, mix the contents of each cup by repeatedly (three to five times) pumping the pipettor in the solution. Use a clean tip in each cup.
- (16) Place cup 0 in the AZ slot and cups 1-4 in slots 1-4 of the AA's autosampler tray.
- (17) Autozero the AA on cup 0.
- (18) Analyze the samples.

- (19) Record the absorbance units and plot these against their calculated concentrations ($\mu\text{g TBTCI/ml}$ in AA cup).³
- (20) If a straight line intersecting 0,0 is not produced, adjust the GF-AAS as necessary and repeat steps 13-19.
- (21) Now set one AA cup per sample into the rack and pipet the following into each cup, being sure to change tips when appropriate:
- 100 μl extract,
 - 200 μl toluene,
 - 600 μl isopropyl alcohol, and
 - 100 μl 0.1 M ammonium dichromate.
- (22) Mix the contents of each cup as described in step 15.
- (23) Place the sample cups into the autosampler tray and note their positions.
- (24) Analyze the samples, and record the absorbance units.
- (25) If any of the extracts give absorbance units in excess of 0.025, these samples will need to be diluted so that they will give readings of ~ 0.020 (i.e., values between 0.010 and 0.025).⁴
- (26) After the dilution factor to obtain ~ 0.020 absorbance has been determined, prepare five cups for the first sample as follows (Note: preparing more than five cups at one time leads to precipitation before the samples are analyzed):

Cup No.	μl of Extract	μl of STD*	μl of Toluene	μl of Isopropyl Alcohol	μl of 0.1 M Ammonium Dichromate
1	0	0	300	600	100
2	X	0	300	600	100
3	X	5	300	600	100
4	X	10	300	600	100
5	X	25	300	600	100

*the sodium hydroxide-treated TBTCI STD ($1-1.5 \mu\text{g TBTCI/ml}$).

X = the amount (diluted or not) of sample needed to give an absorbance between 0.010 and 0.025 with no standard added, i.e., cup 2.

Use fresh pipet tips as necessary and be sure to thoroughly mix the solution in each cup as described in step 15.

- (27) Place cup 0, the blank, in the AZ slot for autozeroing and the remaining cups in consecutive slots, noting their positions.
- (28) Analyze the samples.

- (29) Record the absorbances, and plot absorbance vs. concentration of TBTCI standard in the cups.³
- (30) For any point that does not fall on the line, prepare a fresh cup of extract, standard, and matrix modifier to generate a new point at this concentration.
- (31) Run a linear regression of the data and calculate the amount of tin in the tissue sample ($\mu\text{g Sn/g wet tissue}$).⁵

To obtain a more accurate tin content of the samples (optional):

- (32) Follow step 26, using half as much tissue extract.
- (33) Plot the data as described in step 29.
- (34) Run the linear regression and again calculate the amount of tin in the tissue sample ($\mu\text{g Sn/g wet tissue}$).
- (35) For the two dilutions of extracts analyzed here and in step 26, plot $\mu\text{g Sn/g wet tissue}$ vs. percent dilution of the extract.⁶
- (36) Run a linear regression and find the concentration of tin in the tissue at infinite dilution (i.e., $x = 0$, the y intercept). This should be a better representation of the tin content if there were no matrix effects.

3.4 EXTRACT PREPARATION FOR GC-FID AND GC-MS ANALYSIS

Equipment

Gas Chromatograph (see sections 4.2 and 4.3 for associated equipment and setup parameters)

Glassware

16- X 100-mm PYREX culture tubes, one or three* per sample
 Polypropylene screw caps for culture tubes
 1-ml crimp-seal vials with Teflon-lined septum seals, one per sample
 Miniature champagne columns, one per sample*

Chemical Solvents and Reagents

Internal Standard of dipropyltin dichloride ($[(\text{C}_3\text{H}_7)_2\text{SnCl}_2]$ in toluene (3-4 mg/ml), 10 μl per sample
 2 M hexylmagnesium bromide ($\text{C}_6\text{H}_{13}\text{MgBr}$) in ether (AGFA VENTRON), 100 μl per sample
 1 N sulfuric acid (H_2SO_4), 2 ml per sample
 Sodium chloride (NaCl), ~0.5 g per sample
 Hexane (C_6H_{14}), 3 ml per sample*

*Materials needed for the optional Florisil cleanup procedure. See note below step 6.

Other Materials

Labeling tape, ~1 in. per sample
100-, 500-, and 1000- μ l pipettors with disposable tips
Crimper for crimp-seal vials
Silanized glass wool, ~1 cm² per sample*
Florisil, ~1 ml per sample*
Compressed argon or nitrogen
Mini-vap (SUPELCO), one outlet per sample

Procedure for GC Analysis of TBT and DBT

- (1) Mark 16- X 100-mm culture tubes (one per sample) and place them in a rack.
- (2) Prepare no more than six derivatizations (samples) at a time. Using pipettors and changing pipet tips after each use, add the following to the culture tubes:
 - (a) 10 μ l internal standard (dipropyltin dichloride)
 - (b) 500 μ l of the corresponding, well-mixed extract from the scintillation vial
 - (c) 100 μ l of 2 M hexylmagnesium bromide in ether

Cap the tube immediately after step (c) and mix the solution on a vortex mixer for 30 s, rolling the tube to rinse the inner walls.

- (3) Let the solution react for 20 min. In the meantime, prepare the next five to six samples.
- (4) After the 20-min reaction time, hydrolyze the sample by pipetting 2 ml 1 N sulfuric acid into each tube, capping the tube immediately and placing the tube on the vortex mixer for 30 s.
- (5) Using one disposable funnel per sample, add ~0.5 g (two scoops with a Teflon spatula) sodium chloride to each derivatized extract.
- (6) Recap and mix the tube on a vortex mixer for 30 s.

If the samples are to be analyzed by GC-FID, proceed with step 11. If the samples will be analyzed by GC-MS, either continue at step 7 for immediate analysis or proceed to step 11 for Florisil cleanup.

- (7) Label 1-ml crimp-seal vials (one per sample) with labeling tape.
- (8) After each derivatized sample has separated from the aqueous layer (>5 min), carefully transfer the top layer to the corresponding vial. Be sure to use a fresh pipet tip for each sample and be careful not to transfer any of the bottom layer to the vial.
- (9) Cap the vials, using the crimper.

*Materials needed for the optional Florisil cleanup procedure. See note below step 6.

- (10) Analyze the derivatized extracts by GC-MS and calculate the amount of TBT and DBT as tin in the tissue ($\mu\text{g Sn/g}$ wet tissue).⁷

If the extracts will be cleaned with Florisil, proceed with the following steps (11-25).

- (11) Using forceps and a long-nosed Pasteur pipet, pack a small plug of silanized glass wool into the tip of each champagne column (one column per sample).
- (12) Carefully fill the stem of the column with Florisil, lightly tapping the column as it fills. The level of Florisil should just reach the base of the cup.
- (13) Set each column in a 16- X 100-mm culture tube standing in a rack.
- (14) Pipet 2 ml of hexane onto the top of the packing, letting the first 1 ml partially drain before adding the remaining 1 ml.
- (15) Discard the hexane and culture tubes after the champagne columns have drained.
- (16) Label 16- X 100-mm culture tubes (one per sample) and place one under each champagne column.
- (17) Using a 100- μl pipettor and a fresh tip for each sample, apply 300 μl of the derivatized extract (top layer) from step 8 to the corresponding champagne column.
- (18) After each extract drains into the Florisil, carefully pipet 3 ml of hexane into each cup (1 ml at a time, waiting for the previous rinse to just reach the top of the Florisil before adding the next aliquot).
- (19) Let the hexane completely drain from the Florisil.
- (20) Place each of the culture tubes with the collected eluents under an outlet of the mini-vap, and set the argon or nitrogen regulator for a slow, steady stream.
- (21) When the samples reach dryness, pipet 300 μl toluene into each tube and swirl and roll the tubes to rinse the dried extracts from the inner walls.
- (22) Label 1-ml crimp-seal vials (one per sample) with labeling tape.
- (23) With a fresh tip for each sample, pipet the samples from the culture tubes to their corresponding vials.
- (24) Crimp-seal the vials and analyze the samples by GC-FID.
- (25) Calculate the amount of TBT and DBT as tin in the tissue ($\mu\text{g Sn/g}$ wet tissue).⁷

Note: For GC analyses, be sure that the instruments are calibrated to known TBT and DBT standards and to the particular batch of internal standard which is being added to the samples.

4. SUMMARY OF INSTRUMENTATION

4.1 GF-AAS

Equipment

Perkin-Elmer 5000 Atomic Absorption Spectrophotometer
Perkin-Elmer 8-W Electrodeless Tin Discharge Lamp
Perkin-Elmer EDL Power Supply
Perkin-Elmer AS40
Perkin-Elmer HGA Programmer
Perkin-Elmer Atomic Spectroscopy Data System 10
Perkin-Elmer PR-100 Printer
Perkin-Elmer 056 Strip Chart Recorder
Perkin-Elmer graphite tube with platform
UPC compressed argon
Cooling water - house supply

Parameters

Perkin-Elmer 5000 AAS

time: 8.0 s
Energy: 60
slit Low: 0.7 nm
wavelength: 224.6 nm
Record Absorbance: 1
Average: 3
CV: 1
Atomic Absorption-Background Corrector (AA-BG): ON
Absorbance: ON
Peak Height: ON

Perkin-Elmer AS40

25- μ l sample volume

Perkin-Elmer HGA 500

Step	1	2	3	4
Temperature	100	1000	2700	20
Ramp Time	20	10	0	10
Hold Time	20	0	7	10
Recorder on			-4	
Read			-2	
Base Line			-1	
Internal Flow			0	

Gas

Argon, 50 psi

4.2 GC-FID

Equipment

Hewlett-Packard 5880A Series Gas Chromatograph with Flame Ionization
Detector
Hewlett-Packard GC Terminal
Supelco SPB-1 Fused-Silica Capillary Column, 30 m X 0.25 mm ID with 0.25
μm film thickness
UPC Compressed helium
Compressed hydrogen
Compressed air

Parameters

Oven Temperature Profile and Heated Zones:

Initial Temperature: 50°C
Initial Time: 2.00 min
Program Rate: 30.00°C/min
Final Value: 200°C
Final Time: 7.50 min
Post Value: 280°C
Post Time: 20.00 min
Detector Temperature: 300°C
Injector Temperature: 250°C
Purge Off Time: 0.60 min

Gas

Helium, 30 psi
Hydrogen, 45 psi
Argon, 35 psi

Splitless Injection

Sample Size: 1 μl

Identifying Retention Times

Tributylhexyltin: 8.28 min
Dipropyldihexyltin (internal standard): 8.49 min
Dibutyldihexyltin: 9.83 min

4.3 GC-MS

Equipment

Hewlett-Packard 5890 Gas Chromatograph
Hewlett-Packard 5970 Series Mass Selective Detector with direct capillary inlet
Hewlett-Packard 59970 MS Chemstation

HP-1 Fused-Silica Column, 12 m X 0.2 mm with 0.33 μ m film thickness
UPC Compressed helium

Parameters

Oven Temperature Profile and Heated Zones:

Initial Temperature: 50°C
Initial Time: 3.00 min
Program Rate: 30.00°C/min
Final Temperature: 200°C
Final Time: 6.5 min
Standby Temperature: 225°C
Manual Delays Between Runs: 15 min
Injector Temperature: 250°C
Transfer Line Temperature: 250°C
Purge Off Time: 0.60 min

Selective Ion Mode Acquisition

Electron Multiplier Voltage: 1800 V
m/z: 85, 119, 121, 177, and 191
Dwell: 50
Cycles per second: 3.1

Pressure Settings

Column Head Pressure: 5 psi
Source Pressure: 5 X 10⁻⁵ torr

Splitless Injection

Sample Size: 1 μ l

Identifying Retention Times

Tributylhexyltin: 8.28 min
Dipropyldihexyltin: 8.49 min
Dibutyldihexyltin: 9.83 min

5. SAMPLE CALCULATIONS

Step No.

- 1 Concentration of prepared TBTCI STD ($\mu\text{g/ml}$) = A

B weight of empty flask (g)
C weight of flask with TBTCI added (g)
D volume of toluene added to flask (ml)

$$\frac{C - B}{D} \times 10^6 = A$$

- 2 Dilution of 1° STD to yield 2° STD of ~1-1.5 $\mu\text{g TBTCI/ml}$:

A concentration of 1° STD ($\mu\text{g TBTCI/ml}$)
E desired final concentration of 2° STD (1-1.5 $\mu\text{g TBTCI/ml}$)
F final prepared volume of 2° STD (ml)
G volume of 1° STD to be diluted to F in volumetric flask (ml)

$$\frac{E \times F}{A} = G$$

- 3 Concentration of TBTCI STD in AA cup ($\mu\text{g/ml}$) = H

I concentration of sodium hydroxide-treated STD ($\mu\text{g TBTCI/ml}$)
J amount of STD added to AA cup (μl)
K total volume in AA cup (μl)

$$\frac{I \times J}{K} = H$$

- 4 Dilution of extracts to obtain absorbances of ~0.020:

L amount of extract initially analyzed (μl)
M absorbance reading obtained from L
N dilution factor to obtain an absorbance of ~0.020

$$\frac{M}{0.02} = N$$

either (a) dilute a portion of the original extract to 1/N its concentration and analyze as before, or (b) prepare an AA cup as before, but add 1/N of L.

For example:

100 μl of extract gave an absorbance reading of 0.042, but an absorbance of ~0.020 is wanted. Thus

L = 100 μl
M = 0.042

and from above:

$$N = \frac{0.042}{0.02} \sim 2$$

Therefore either (a) dilute some of the extract to one-half its concentration with toluene in a clean 16- X 100-mm culture tube and analyze 100 μ l of the diluted extract as described in step 21, section 3.3, or (b) analyze one-half as much of the original extract by preparing the sample as in step 21, except use 50 μ l of extract instead of 100 μ l.

Whichever method of dilution is chosen, be sure that the AA cup solution ratio is ~3:6:1 of toluene, isopropyl alcohol, and 0.1 M ammonium dichromate, respectively.

- 5 Concentration of tin extracted as TBT from the tissue (μ g Sn/g wet tissue), as determined by GF-AAS = P

From the linear regression of absorbance vs. concentration of TBTCI STD (μ g TBTCI/ml) in the AA cups, solve the equation for $y = 0$. This is (-) the concentration of TBT in the cup which contains the extract but no added standard. Call this value Q.

- N dilution factor calculated from step 4, if there is one
R amount of extract added to AA cup (μ l)
S total volume in AA cup (μ l)
T concentration of TBTCI in the extract (μ g/ml), calculated below
U amount of toluene used to reconstitute dried extract (ml)
V wet weight of tissue extracted (g)
W concentration of TBT extracted from the tissue (μ g TBTCI/g wet tissue), calculated below
Y fraction of TBTCI as tin, 0.37

$$\frac{S \times Q}{R} = T$$

$$\frac{T \times N \times U}{V} = W$$

$$W \times Y = P$$

- 6 Percent dilution of the extract = Z

- R amount of extract added to AA cup (μ l)
S total volume in AA cup (μ l)
N dilution factor calculated from step 4, if there is one

$$\frac{R}{N \times S} \times 100 = Z$$

- 7 Concentration of tin extracted as TBT and DBT from tissue (μ g Sn/g wet tissue), as determined by GC:

- U amount of toluene used to reconstitute dried extract (ml)

V wet weight of tissue extracted (g)
 Y fraction of TBTCI as tin, 0.37
 EE fraction of DBTCI as tin, 0.40
 AA concentration of TBT in derivatized extract analyzed by GC (μg TBTCI/ml)
 BB concentration of DBT in derivatized extract analyzed by GC (μg DBTCI/ml)
 CC total volume in vial (μl)
 DD amount of extract in vial (μl)
 FF concentration of tin extracted from tissue as TBT ($\mu\text{g Sn/g}$ wet tissue)
 GG concentration of tin extracted from tissue as DBT ($\mu\text{g Sn/g}$ wet tissue)

$$\frac{AA \times CC \times U \times Y}{DD \times V} = FF$$

$$\frac{BB \times CC \times U \times EE}{DD \times V} = GG$$

6. CONCLUDING REMARKS

Tissue of marine organisms can be effectively analyzed by GF-AAS, GC-FID, or GC-MS for organotins as described. However, experience at NOSC with these procedures gives rise to the following observations and theories:

- (1) Tin values obtained by GF-AAS may be artificially low or high due to unknown compounds in the tissue extracts that act as Sn signal suppressors or enhancers.
- (2) There is a possibility for breakdown of TBT in the extracts prepared for GF-AAS analysis. In a simple solvent system, different butyltin species give different AAS responses per μg Sn analyzed. However, by adding the ammonium dichromate matrix modifier, the AAS response to Sn is better smoothed for the varying butyltin species. Thus, breakdown products should cause a minimal problem when quantifying Sn content of tissue extracts. In addition, the ammonium dichromate enhances the GF-AAS signal to Sn so that samples with low levels of Sn are more easily quantified.
- (3) Some alkylmagnesium bromide reagents can be contaminated with TBT and/or DBT. This contamination can lead to significant introduction of variable amounts of TBT and DBT into the samples, thus causing quantification problems, especially in samples containing low levels of these compounds. It is therefore recommended that before using a particular batch of derivatizing agent on valuable samples, it be checked for purity by running blanks of the stock.

Work will continue to correct for these analytical inefficiencies.

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ANALYTICAL CHEMISTRY

QUALITY ASSURANCE AND QUALITY CONTROL

PROTOCOLS, CRITERIA, AND CORRECTIVE ACTION

FOR THE

ESTUARINE ECOLOGICAL RISK ASSESSMENT

AT NAVAL SHIPYARD PORTSMOUTH, KITTERY, ME

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PREFACE

This document provides guidance for conducting field and laboratory quality assurance and quality control (QA/QC) protocols, criteria and corrective action for the Estuarine Ecological Risk Assessment (ERA) for Naval Shipyard Portsmouth (NSYP). The data quality objectives for conducting an ecological risk assessment requires the use of field and laboratory methods that are not currently officially approved by regulatory agencies. Therefore QA/QC procedures are required that will assure that high-quality, scientifically sound data are obtained during the study. The purpose of this document is to provide the QA/QC framework for implementing performance based protocols, criteria and corrective action for field and laboratory activities to be undertaken during the study. Many of the ERA-NSYP data quality requirements are similar the requirements identified for the Environmental Protection Agency's (EPA) Environmental Monitoring and Assessment Program (EMAP) Near Coastal Demonstration Project (NCDP). As such this document has been adapted from the QA/QC guidance developed for the EMAP-NCDP in order to benefit from EPA's existing technical and administrative experience (Valente and Strobel 1991).

Complete and detailed protocols for field and laboratory measurements can be found in Strobel et al. (1991), Graves et al. (in preparation), and Mueller et al. (1991). Specific quality assurance procedures for the laboratory are provided in a companion appendix (Appendix E, Ceimic Corp. 1991). Critical features of the QA/QC procedures to be followed during the ERA at NSYP are presented below.

1.0 CHEMICAL ANALYSIS OF SEDIMENT AND TISSUE SAMPLES

For analysis of the parts-per-billion levels of organic and inorganic contaminants in estuarine sediments and tissue (fish, invertebrates, and plants), no procedure has been officially approved by the regulatory agencies. The recommended analytical methods for the purposes of this project are those prescribed by NOAA Status and Trends Program (MacLeod et al. 1985, Krahn et al. 1988), the Puget Sound Estuary Program (TetraTech 1986a and 1986b), and the Risk Assessment Pilot Study for NCBC Davisville, RI (Gleason and Mueller 1989, Mueller et al. 1991, Munns et al. 1991). These procedures have been recommended to meet the data quality objectives for the National Status and Trends Program, the Puget Sound Estuary Program, and a CERCLA assessment for NCBC Davisville, which were conducted by multiple agencies, including EPA, NOAA and the US Navy. These programs do not specifically require that particular analytical methods always be followed, but rather that participating laboratories demonstrate proficiency through routine analysis of standard or certified reference materials (SRMs or CRMs)¹ or similar types of accuracy-based materials. Through the application of this concept, the analytical laboratory selected for the ERA-NSYP will participate in on-going performance evaluation exercises conducted throughout the study, both to demonstrate initial capability (i.e., prior to the analysis of actual samples) and on a continuous basis throughout the project. The laboratory will be required to initiate corrective actions if their performance falls below certain predetermined minimal standards, described in later sections.

The data quality objectives for this project were developed with the understanding that the data will of sufficient quality to address research and monitoring activities in a scientifically sound manner. Because high quality low detection limits are required and the research nature of much of the proposed work a QA/QC plan is needed that will expand on areas not addressed by the USEPA Contract Laboratory Program. Accordingly, the procedures outlined in this document should be viewed as additions and expansions to CLP protocols in those areas which are not addressed by CLP methods. In addition, it is

¹ Certified Reference Materials are samples containing precise concentrations of chemicals, accurately determined by a variety of technically valid procedures and accompanied by a certificate or other documentation issued by a certifying body (agencies such as the National Research Council of Canada (NRC), USEPA, US Geological Survey, etc.). Standard Reference Materials (SRMs) are CRMs issued by the National Institute of Standards and Technology (NIST), formerly the National Bureau of Standards.

the philosophy of this project that as long as proper QA/QC requirements are implemented and comparable analytical performance on standard materials is demonstrated, multiple procedures for the analysis of different compound classes used by different laboratories should yield comparable results. Based on this assumption, the QA/QC requirements for the analysis of contaminants in sediments and tissue will provide special emphasis on a performance-based program, involving continuous laboratory evaluation through the use of accuracy-based materials (e.g., certified standard reference materials and laboratory control materials), laboratory fortified sample matrices, laboratory reagent blanks, calibration standards, and laboratory and field replicates. The conceptual basis for the use of these quality control samples is presented below. In all other areas, not explicitly addressed by this document (instrument tuning, chain-of-custody, data validation, etc.), standard CLP protocols will apply.

1.1 General QA/QC Requirements

The guidance provided in the following sections is based largely on the protocol developed for the Status and Trends Program (Krahn et al. 1988, MacLeod et al. 1985), the Puget Sound Estuary Program (TetraTech 1986a and 1986b) and the Risk Assessment Pilot Study for NCBC Davisville (Gleason and Mueller 1989, Mueller et al. 1991, Munns et al. 1991); it is applicable to low parts-per-billion analyses of both sediment and tissue samples unless otherwise noted. QA/QC requirements are the foundation of this protocol because they provide information necessary to assess the comparability of data generated by different laboratories and different analytical procedures. It should be noted that the QA/QC requirements specified in this plan represent the minimum requirements for any given analytical method. Additional requirements which are method-specific should always be followed, as long as the minimum requirements presented in this document have been met.

Data for all QA/QC variables must be submitted by the laboratory as part of the data package. Program managers and project coordinators must verify that requested QA/QC data are included in the data package as supporting information for the summary data. A detailed QA/QC review of the entire data package (especially original quantification reports and standard calibration data) will be conducted by QA personnel at the EPA-ERL. The QA/QC data will be used initially to document the accuracy and precision of individual measurement processes, and ultimately to assess comparability among different laboratories.

The analysis results for the various QA/QC samples should be used directly by the analytical laboratory to determine when warning and control limits have been exceeded and corrective actions must be taken. Warning limits are numerical criteria that serve as flags to data reviewers and data users. When a warning limit is exceeded, the laboratory is not obligated to halt analyses, but the reported data may be qualified during subsequent QA/QC review. Control limits are numerical data criteria that, when exceeded, require specific corrective action by the laboratory before the analyses may proceed. Warning and control limits and recommended frequency of analysis for each QA/QC element or sample type required for the ERA-NSYP project are summarized in Table 1. Descriptions of the use, frequency of analysis, type of information obtained, and corrective actions for each of these QA/QC sample types or elements are provided in the following sections.

1.2 Initial Calibration

Equipment must be calibrated before any samples are analyzed, after each major equipment disruption, and whenever on-going calibration checks do not meet recommended control limit criteria (Table 1). Summary data documenting initial calibration and any events requiring recalibration and the corresponding recalibration data must be included with the analytical results. All standards used for initial calibration must be obtained from a single source and should be traceable to a recognized organization for the preparation of QA/QC materials (e.g., National Institute of Standards and Technology, Environmental Protection Agency, etc.). Calibration curves must be established for each element and

batch analysis from a calibration blank and a minimum of three analytical standards of increasing concentration, covering the range of expected sample concentrations. The calibration curve must be established prior to the analysis of samples. Only data which results from quantification within the demonstrated working calibration range may be reported by the laboratory; samples outside the calibration range should be diluted or concentrated, as appropriate, and reanalyzed.

TABLE 1. KEY ELEMENTS FOR QUALITY CONTROL OF ESTUARINE ECOLOGICAL RISK ASSESSMENT CHEMICAL ANALYSES.

Element or Sample Type	Recommended Warning Limit	Recommended Control Limit	Recommended Frequency
1. Performance Evaluation (PE): (Prior to Analysis of Samples):			
- Instrument Calibration	NA ^a	NA ^a	Initial
- Documentation of Detection Limits	NA ^a	NA ^a	Per analyte for each matrix
- Blind Analysis of PE organic samples	80%-120% ^b	70%-130%	Initial
PE inorganic samples	90%-110%	85%-115%	
2. On-going Demonstration of Capability:			
- Analysis of Laboratory Control Material:			
organic analyses	80%-120% ^b	70%-130%	One per batch or one every 15 samples
inorganic analyses	90%-110%	85%-115%	
- Analysis of Standard Reference Material	same as above	same as above	SRMS included in every batch

Continued on following page

^a Not required for initial calibration at outset of project

^b Percent of true value

TABLE 1. (Continued)

Element or Sample Type	Recommended Warning Limit	Recommended Control Limit	Recommended Frequency
3. Calibration Check using Calibration Standard	NA	within 15% of initial calibration on average for all analytes, not to exceed 25% for any one analyte	Beginning and end of batch, every 10 samples
4. Laboratory Reagent Blank	between MDL and 3 times MDL	greater than 3 times MDL	One per batch
5. Laboratory Fortified Sample Matrix	$\pm 50\%^c$	not specified	At least 5% of total samples
6. Laboratory Duplicate	NA	$\pm 30\%$ (RPD) ^d	One per batch
7. Field Duplicates (Field Splits)	NA	NA	5% of total no. of samples
8. Internal Standards (Surrogate Analytes)	Lab develops its own		Each sample
9. Injection Internal Standards	Lab develops its own		Each sample
10. Interlaboratory Calibration	Subject to Review by Project Officers and Project QA Officers		5% of sample by matrix

^c Percent recovery^d RPD = Relative percent difference

1.3 Initial Documentation of Detection Limits

For the purpose of clarity this document will distinguish between two kinds of "limits" of detectability: the Method Detection Limit (MDL) and the Limit of Quantitation (LOQ). Complete definitions and detailed discussions of these two terms and their practical applications are provided in Keith et al. (1983) and in Keith (1991a and 1991b). Brief descriptions of these terms and their use for this program are provided in the following paragraphs.

The MDL represents a quantitative estimate of low-level response detected at the maximum sensitivity of a method. The Code of Federal Regulations (40 CFR Part 136) gives the following rigorous definition: "the MDL is the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero and is determined from analysis of a sample in a given matrix containing the analyte." Confidence in the apparent analyte concentration increases as the analyte signal increases above the MDL.

For the initial documentation of detection limits, the analytical laboratory must establish and report an MDL for each analyte of interest in each matrix of interest (sediment or tissue) prior to the analysis of field samples. Each laboratory is required to follow the procedures specified in 40 CFR Part 136 to calculate MDLs for each analytical method employed. In order to ensure comparability of results, MDL target values have been established for the ERA for NSYP (Table 2). The initial MDLs reported should be equal to or less than these specified target values before the analysis of field samples may proceed. It is recognized that the initial MDL is a statistically-derived, empirical value that subsequently may vary in actual samples as a function of sample size, matrix, percent moisture, etc. Therefore the laboratory should periodically (i.e. at least once a year) re-evaluate its MDLs for the analytical methods used and the sample matrices typically encountered.

Keith (1991a) defines the LOQ as the level above which quantitative results maybe obtained with a specified degree of confidence. In practice, the LOQ usually represents a reported concentration level above which there is a high technical confidence in the quantified result (i.e., there is a low probability of either a false positive or false negative result at the LOQ). The LOQ is different from, and more difficult to reach than, simply measuring the presence or absence of an analyte (Keith 1991b). For each analyte, the recommended value for the LOQ of 10 times the standard deviation used in calculating the MDL (Keith 1991a and 1991b). Sample concentrations equal to or greater than the LOQ may be reported without qualification.

1.4 Performance Evaluation of Initial Blind Analysis of Reference Material

A representative sample matrix which is homogenous and contains known concentrations of the analytes of interest will be provided to the analytical laboratory. These Performance Evaluation (PE) samples of sediments and tissues will be used to evaluate laboratory performance prior to the analysis of field samples. The initial analysis of whatever reference material is provided must be blind (i.e., the laboratory must not know the concentrations of the analytes of interest). The laboratory's performance generally will be considered acceptable if its submitted values are within $\pm 30\%$ (for organic analyses) and $\pm 15\%$ (for inorganic analyses) of the actual or certified concentration of each analyte in the sample. If any of the values resulting from the initial analysis are outside the control limit, the laboratory will be required to repeat the analysis until the control limit is met, prior to the analysis of real samples.

TABLE 2. Target analytes for chemical analysis.

A. Organic compounds.		(Dry weight for sediment and biota) Target Detec- tion Limit
Analyte	Sample Matrix	
Volatile Organic Compounds	seep water	0.1 µg/L
vinyl chloride	trans-1,3-dichloropropene	
1,1-dichloroethene	tetrachloroethene	
methylene chloride	chlorobenzene	
trans-1,2-dichloroethene	bromoform	
chloroform	1,1,2,3-tetrachloroethane	
1,1,1-trichloroethane	1,3-dichlorobenzene	
carbon tetrachloride	methyl-t-butyl ether	
1,2-dichloroethane	benzene	
trichloroethene	toluene	
1,2-dichloropropane	ethylbenzene	
bromodichloromethane	m,p-xylene	
2-chloroethylvinyl ether	o-xylene	
cis-1,3-dichloropropene	1,2-dichlorobenzene	
Polycyclic Aromatic Hydrocarbons	seep water	1-5 µg/L
	sediment	1-5 ng/g
	biota	10-20 ng/g
anthracene	phenanthrene	
benz(a)anthracene	C ₁ alkyl phenanthrenes + anthracenes	
benzo(a)pyrene	C ₂ alkyl phenanthrenes + anthracenes	
benzo(e)pyrene	C ₃ alkyl phenanthrenes + anthracenes	
chrysene	C ₄ alkyl phenanthrenes + anthracenes	
dibenz(a,h)anthracene	pyrene	
fluoranthene	sum of benzo(g,h,i)perylene	
fluorene	indeno(1,2,3-cd)pyrene	
perylene		
Chlorinated Pesticides	seep water	0.6 ng/L
	sediment	0.6 ng/g
	biota	0.6 ng/g
aldrin	alpha-chlordane	
trans-nonachlor	Heptachlor	
Hepachlor epoxide	hexachlorobenzene	
Lindane (gamma-BHC)	Mirex	
o,p'-DDD	p,p'-DDD	
o,p'-DDE	p,p'-DDE	
o,p'-DDT	p,p'-DDT	
Polychlorinated Biphenyl Congeners	seep water	1 µg/L
(Congener number and position of chlorines)	sediment	0.5 ng/g
	biota	0.5 ng/g
8 (2,4')	18 (2,2',5)	
28 (2,4,4')	52 (2,2',5,5')	
44 (2,2',3,5')	66 (2,3',4,4')	
101 (2,2',3,5,5')	118 (2,3',4,4',5)	
153 (2,2',4,4',5,5')	105 (2,3,3',4,4')	
138 (2,2',4,4',5,5')	187 (2,2',3,4',5,5',6)	
128 (2,2',3,3',4,4')	180 (2,2',3,4,4',5,5')	
170 (2,2',3,3',4,4',5)	195 (2,2',3,3',4,4',5,6)	
206 (2,2',3,3',4,4',5,5',6)	209 (2,2',3,3',4,4',5,5',6,6')	

TABLE 2. Continued.

B. Inorganic compounds.

Analyte	Matrix	Target MDL	
Aluminum	seep water	75.0	$\mu\text{g/L}$
Aluminum	sediment	Not Specified(NS)	
Aluminum	tissue	NS	
Arsenic	seep water	3.0	$\mu\text{g/L}$
Arsenic	sediment	1.1	$\mu\text{g/g}$ (DW)
Arsenic	tissue	4.3	$\mu\text{g/g}$ (DW)
Cadmium	seep water	0.2	$\mu\text{g/L}$
Cadmium	sediment	0.35	$\mu\text{g/g}$ (DW)
Cadmium	tissue	0.055	$\mu\text{g/g}$ (DW)
Chromium	seep water	3.0	$\mu\text{g/L}$
Chromium	sediment	3.16	$\mu\text{g/g}$ (DW)
Chromium	tissue	0.28	$\mu\text{g/g}$ (DW)
Copper	seep water	0.7	$\mu\text{g/L}$
Copper	sediment	1.25	$\mu\text{g/g}$ (DW)
Copper	tissue	5.0	$\mu\text{g/g}$ (DW)
Iron	seep water	20.0	$\mu\text{g/g}$
Iron	sediment	NS	
Iron	tissue	NS	
Lead	seep water	3.0	$\mu\text{g/L}$
Lead	sediment	1.2	$\mu\text{g/g}$ (DW)
Lead	tissue	0.6	$\mu\text{g/g}$ (DW)
Manganese	seep water	0.5	$\mu\text{g/L}$
Manganese	sediment	NS	
Manganese	tissue	NS	
Mercury	seep water	5.0	$\mu\text{g/L}$
Mercury	sediment	0.007	$\mu\text{g/g}$ (DW)
Mercury	tissue	0.036	$\mu\text{g/g}$ (DW)
Nickel	seep water	3.0	$\mu\text{g/L}$
Nickel	sediment	1.08	$\mu\text{g/g}$ (DW)
Nickel	tissue	0.73	$\mu\text{g/g}$ (DW)
Silver	seep water	3.0	$\mu\text{g/L}$
Silver	sediment	0.04	$\mu\text{g/g}$ (DW)
Silver	tissue	0.037	$\mu\text{g/g}$ (DW)
Tin	seep water	3.0	$\mu\text{g/L}$
Tin	sediment	1.75	$\mu\text{g/g}$ (DW)
Zinc	seep water	0.1	$\mu\text{g/L}$
Zinc	sediment	2.15	$\mu\text{g/g}$ (DW)
Zinc	tissue	11.65	$\mu\text{g/g}$ (DW)
Butyltins	sediment	2.0	$\mu\text{g/g}$
	blota	2.0	$\mu\text{g/g}$
	monobutyltin		
	dibutyltin		
	tributyltin		

1.5 Routine Analysis of SRM's and Laboratory Control Materials

Reference Materials (SRMs or CRMs) generally are considered one of the most useful QC samples for assessing the accuracy of a given analysis (i.e., the closeness of a measurement to its true value). Reference Materials can be used to assess accuracy because they have "certified" concentrations of the analytes of interest, as determined through replicate analyses by a reputable certifying agency using two independent measurement techniques for verification. In addition, the certifying agency may provide "noncertified" or "informational" values for other analytes of interest. Such values are determined using a single measurement technique, which may introduce unrecognized bias. Therefore, non-certified values must be used with caution in assessing the accuracy of a laboratory method which differs from the one used by the certifying agency.

A laboratory control material is similar to a Certified Reference Material in that it is a homogeneous matrix which closely matches the samples being analyzed, and the concentrations of certain analytes of interest are known with reasonable accuracy (i.e., as a result of a statistically-valid number of replicate analyses by one or several laboratories). In practice, this material is not officially certified, but it can be used by laboratories to assess both accuracy and precision (i.e., batch-to-batch consistency). Routine analysis of laboratory control materials or certified reference materials thus represents a particularly vital aspect of the "performance-based" philosophy.

For the analysis of samples for the ERA-NSYP, one SRM, CRM or laboratory control material should be analyzed along with each batch of samples (Table 1). The concentrations of the target analytes should be known to the analyst and should be used to provide an immediate check on accuracy for each batch of samples. If the values are outside the control limits (Table 1), the data for the entire batch of samples are suspect. Calculations and instruments should be checked; the SRM or CRM should be reanalyzed to confirm the results. If the values are still outside the control limits in the repeat analysis, the laboratory is required to determine the source(s) of the problem and repeat the analysis of that batch of samples until control limits are met, before continuing with further sample analyses.

Analysis results for reference materials and laboratory control materials also should be recorded on control charts to monitor laboratory precision from batch to batch. This is particularly important in situations where certified or "true" concentrations are not available for all the analytes of interest in a particular control material, or where reference material concentrations are given only as "non-certified" values. In the latter case, a laboratory may find that a "method bias" prevents it from meeting the 70 to 130 percent recovery control limit for one or more analytes of interest in a particular SRM or CRM. In such instances, the laboratory should be able to demonstrate (through the use of control charts) that its results are consistent from batch-to-batch for each analysis of a particular reference material or laboratory control material (i.e., wildly fluctuating results are not acceptable). The results of the reference material or laboratory control material analysis should never be used by the laboratory to "correct" the data for a given sample batch. Instead, a special data qualifier code "p" (see section 1.13) may be used in those instances where the laboratory is able to demonstrate a consistent method bias in quantifying one or more analytes having non-certified concentrations in the SRM or CRM.

1.6 Calibration Check

The initial instrument calibration is checked through the analysis of a calibration standard. The calibration standard solution used for the calibration check should be obtained from a different source, if possible than the initial calibration standards, so that it can provide an independent check both on the calibration and the accuracy of the standard solutions. Analysis of the calibration standard should occur at the beginning of a sample set, once every 10 samples or every two hours during a run, and after the last analytical sample.

If the control limit for analysis of the calibration standard (Table 1) is not met, the initial calibration will have to be repeated. If possible, the samples analyzed before the calibration check that failed the control limit criteria should be reanalyzed following the re-calibration. The laboratory should begin by reanalyzing the last sample analyzed before the calibration standard which failed. If the relative percent difference (RPD) between the results of this reanalysis and the original analysis exceeds 30 percent, the instrument is assumed to have been out of control during the original analysis. If possible, reanalysis of samples should progress in reverse order until it is determined that there is less than 30 RPD between initial and reanalysis results. If it is not possible or feasible to perform reanalysis of samples, all earlier data (i.e., since the last successful calibration control check) should be flagged. In this case, the laboratory should prepare a narrative explanation to accompany the submitted data.

1.7 Laboratory Reagent Blank

Laboratory reagent blanks (commonly called method blanks) are used to assess laboratory contamination during all stages of sample preparation and analysis. For both organic and inorganic analyses, one reagent blank should be run in every sample batch or for every 12-hour shift, whichever is more frequent. Warning and control limits for blanks (Table 1) are based on the laboratory's method detection limits as documented prior to the analyses of samples (Table 2). A reagent blank concentration between the MDL and 3 times the MDL should serve as a warning limit requiring further investigation based on the best professional judgement of the analyst(s). A reagent blank concentration equal to or greater than 3 times the MDL requires definitive corrective action to identify and eliminate the source(s) of contamination.

1.8 Internal Standards

Internal standards (commonly referred to as surrogate spikes or surrogate analytes) are compounds chosen to simulate the analytes of interest. The internal standard represents a reference against which the signal from the analytes of interest is compared directly for the purpose of quantification. Internal standards must be added to each sample, including QA/QC samples, prior to extraction, purging, or digestion. The reported concentration of each analyte should be adjusted to correct for the recovery of the internal standard, as is done in the NOAA National Status and Trends Program. The internal standard recovery data therefore should be carefully monitored; the laboratory should report the absolute amounts and the percent recovery of the internal standards along with the target analyte data for each sample. If possible, isotopically-labeled analogs of the analytes should be used as internal standards.

Recommended control limits for internal standard recoveries are not specified. Instead, the laboratory must set its own warning and control limits based on the experience and best professional judgement of the analyst. It is the responsibility of the analyst to demonstrate that the analytical process is always "in control" (i.e., highly variable internal standard recoveries for repeat analyses of the same reference material, or for laboratory duplicates, are not acceptable).

1.9 Injection Internal Standards

For gas chromatography (GC) analysis, injection internal standards (also termed "GC standards") are added to each sample just prior to injection to enable optimal quantification, particularly of complex extracts subject to retention time shifts relative to the analysis of standards. Injection internal standards are essential if the actual recovery of the internal standards added prior to extraction is to be calculated. The injection internal standards can be used to detect and correct for problems in the GC injection port or other parts of the instrument. The compounds used as injection internal standards must be different from those already used as internal standards. The analyst must monitor injection internal standard retention times and recoveries to determine if instrument maintenance or repair, or changes in analytical

procedures, are indicated. Corrective action should be initiated based on the experience of the analyst and not because warning or control limits were exceeded. Instrument problems that may have affected the data or resulted in the reanalysis of the sample must be documented in the analyst's logbook and on the raw data report. Justification for reanalysis must be submitted with the data package.

1.10 Laboratory Fortified Sample Matrix

A laboratory fortified sample matrix (commonly called a matrix spike) will be used to evaluate the effect of the sample matrix on the recovery of the compound(s) of interest. A minimum of 5% of the total number of samples submitted to the laboratory should be selected at random for analysis as laboratory fortified samples. The compounds used to fortify samples should include a wide range of representative analyte types. These compounds should be added at 5 to 10 times their MDLs as previously calculated by the laboratory (see Section 1.3).

The recovery data for each fortified compound, which should be reported along with the rest of the data for each sample, ultimately will provide a basis for determining the prevalence of matrix effects in the samples analyzed during the project. If the percent recovery for any analyte is less than the recommended warning limit of 50 percent, the chromatograms and raw data quantitation reports should be reviewed. If an explanation for a low percent recovery value is not discovered, the instrument response may be checked using a calibration standard. Low matrix spike recoveries may be a result of matrix interferences and further instrument response checks may not be warranted, especially if the other laboratory QC samples indicate that the analysis for that batch of samples was in control. An explanation for low percent recovery values for matrix spike results should be discussed in a cover letter accompanying the data package. Corrective actions taken and verification of acceptable instrument response must be included.

1.11 Laboratory Duplicates

One sample per batch should be split in the laboratory and analyzed in duplicate to provide an estimate of analytical precision. Duplicate analyses also are useful in assessing potential sample heterogeneity and matrix effects. The recommended control limit for analysis of laboratory duplicates is a relative percent difference (RPD) of $\pm 30\%$ for each analyte of interest (Table 1), calculated as follows:

$$RPD = \frac{(C1 - C2) \times 100\%}{(C1 + C2)/2}$$

where: C1 is the larger of the duplicate results for a given analyte
C2 is the smaller of the duplicate results for a given analyte

If results for a significant number of analytes fall outside the control limit, calculations and instruments should be checked. A replicate analysis may be required to confirm the results. If results continue to exceed the control limit, subsequent corrective action is at the discretion of the program manager or QA officer, because matrix effects or incomplete homogenization (either in the field or laboratory) may be contributing factors. A discussion of the results of duplicate sample analysis should include probable sources of laboratory error, evidence of matrix effects, and an assessment of natural sample variability. Data outside the control limit may be flagged pending QA review of the probable laboratory or field sources of variation.

1.12 Sample Analysis

An example of the minimum (5%) QA/QC samples required for the analysis of a hypothetical batch of 20 field samples is provided in Table 3. In addition to extracting the 20 field samples an additional 4 samples consisting of 1 SRM sample, 1 Laboratory Duplicate sample, 1 Fortified Sample Matrix, and 1 Laboratory Reagent Blank must also be extracted. These additional samples are treated exactly the same as the field samples during the analysis. Prior to actual analysis 4 Calibration Check samples are added to the batch; one at the beginning of the batch, one after every tenth analysis, and one at the end of the batch resulting in a total of 28 analysis. Results from all analysis are included in the data report for the batch.

Table 3. Example of the minimum (5%) QA/QC samples required for the analysis of a hypothetical batch of 16 field samples.

Sample	Number	Description
S ₁ , S ₂ , S ₃ , ..., S ₁₆	16	Field Samples
SRM	1	Standard Reference Material
LD	1	Laboratory Duplicate
LFSM	1	Laboratory Fortified Sample Matrix
BLANK	1	Reagent Blank
Total ES ₁ , ..., ES ₂₀	20	Extracted Samples

EXTRACTION

SEQUENCE OF ANALYSIS

Sample	Number	Description
CC ₁	1	Calibration Check
ES ₁ , ..., ES ₁₀	10	Extracted Samples
CC ₂	1	Calibration Check
ES ₁₁ , ..., ES ₂₀	10	Extracted Samples
CC ₃	1	Calibration Check
Total	23	Analytical Analyses

1.13 Sample Storage and Handling

The procedures for field collection of samples is documented in other sections and Appendices of the ERA-NSYP Work Quality Assurance Project Plan. Sediments and tissue samples are to be kept frozen (-10° C) until just prior to analysis. For the determination of analytes listed in Table 2, with the exception of VOCs, no specific holding time limitation is necessary for samples stored in this manner (Gleason and Mueller 1989). Sample handling requirements for other samples to be analyzed for the ERA-NSYP (water samples for metals, seep samples for VOCs, and dried algal samples for metals) are documented in other sections of the work plan and will be provided to the laboratory as required.

When sediments and tissues are to be analyzed they will be thawed to room temperature and homogenized. Any split samples required for interlaboratory calibration will be subsampled from the homogenate. The split samples will then be refrozen for delivery to ERLN. Aliquots of the homogenized matrix will be selected for analysis and any remaining homogenate should be immediately refrozen and archived for future analysis. Extractions of the aliquots should be performed within 24 hr of thawing. The extracts can be stored up to six months, if they are kept at 4°C (Gleason and Mueller 1989), before analysis. Special instructions for homogenizing tissue samples (whole or dissected) and any other special sample handling procedure will be provided in accordance to the work plan SOPs for the ERA-NSYP. All sample inventory, sample information, and status will be maintained in a database system and documented on chain-of-custody logs in accordance with CLP QA/QC guidance.

1.14 Analytical Chemistry Data Reporting Requirements

As previously indicated, data for all QA/QC variables (e.g., SRMs or CRMs, calibration check samples, blanks, laboratory duplicates, etc.) must be submitted by the laboratory as part of the data package for each batch of samples analyzed. The QA/QC results and associated data will be subject to review by the Project Officers, QA Officer, or their designee(s). The laboratory is responsible for assigning data qualifier codes (i.e. "flags") to the data prior to submission; allowable codes are given in Table 3. This list of codes is consistent with that used in the NOAA National Status and Trends Program, with the addition of the "p" code related to reference material analysis results. These are the only codes which should be used by the laboratory. Any other qualifications of the data which the laboratory feels are not covered by the allowable codes (e.g., minor exceedance of a control limit where sample re-analysis was not justified or not possible) should be explained in a cover letter accompanying the data. In these instances, the QA Officer will decide if additional qualification of the data is needed in the project database.

TABLE 4. DATA QUALIFIER CODES USED BY THE ERA-NSYP PROJECT

<u>Code</u>	<u>Description</u>
a	analyte was not detected at the MDL shown
b	reported value is below the LOO (i.e., estimate)
c	not reported due to matrix interference
d	not quantified
e	not reported
h	quantification based on alternate internal standard
j	analysis performed with selected ion monitoring
p	value shown may be biased as determined by recovery of analyte in reference material

2.0 References

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